THE ROLE AND LIFE CYCLE OF CYTOSOLIC DNA IN CANCER

KOO XING'ER CHRISTINE

B.Sc. (Hons), Nanyang Technological University

A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

NUS GRADUATE SCHOOL FOR INTEGRATIVE

SCIENCES AND ENGINEERING

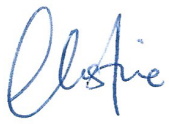
NATIONAL UNIVERSITY OF SINGAPORE

2014

Declaration

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information, which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



Koo Xing'er Christine

15th August 2014

Publications

Peer-reviewed articles

1. Koo CX, Kobiyama K, Shen YJ, LeBert N, Ahmad S, Khatoo M, Aoshi T, Gasser S and Ishii KJ. RNA Polymerase III Regulates Cytosolic RNA:DNA Hybrids and Intracellular MicroRNA Expression. *Manuscript submitted.*

2. Lam AR, Koo CX, Tey CS, Shen YJ, Karim SAM, Akira S, Ishii KJ and Gasser S. The DNA sensor ZBP1 counters ATM-mediated tumour suppression of Eμ-Myc-induced B-cell lymphomagenesis. *Manuscript submitted.*

3. Kuroda E, Ohata K, Ozasa K, Koo CX, Horie M, Morimoto Y, Nakajima S, Kabashima K, Ziegler SF, Coban C, Kobiyama K, Aoshi T and Ishii KJ. Aerosol particulates kill alveolar macrophages to release DNA and IL-1-alpha that mediate lung inflammation and IgE production. *Manuscript submitted.*

4. Shen YJ, Le Bert N, Chitre AA, Koo CX, Nga XH, Ho SSW, Ishii KJ, Raulet DH, and Gasser S. Genome-derived cytosolic DNA mediates type I interferon-mediated rejection of B-cell lymphoma cells. *Manuscript under preparation for resubmission.*

5. Lam AR, Le Bert N, Ho SSW, Shen YJ, Tang LFM, Xiong, GM, Croxford JL, Koo CX, Ishii KJ, Akira S, Raulet DH and Gasser S. (2014) RAE-1 ligands for the NKG2D receptor are regulated by STING-dependent DNA sensor pathways in lymphoma. Cancer Res *74*: 2193-2203.

Book Chapters

1. Shen YJ, Lam AR, Ho SSW, Koo CX, Le Bert N and Gasser S. Cancer pathogenesis and DNA sensing, in Ishii KJ and Choon KT, *Biological DNA sensor: the impact of nucleic acid acids on diseases and vaccinology* (pp.205-229). Academic Press. ISBN: 9780124047327.

Acknowledgements

I would like to thank NGS for the generous financial support over the course of my PhD education, including supporting my research attachment overseas. I would like to express my gratitude to my supervisor Dr. Stephan Gasser for your patient guidance and strong support in all aspects of my PhD life. To my supervisor in Japan, Dr. Ken J. Ishii, thank you for your hospitality and liberty for me to work in your lab. I would also like to express my gratitude towards my TAC members for taking their time to provide me with feedback on the project. I would also like to thank Dr. Ludovic Croxford, Dr. Cevayir Coban, Dr. Taiki Aoshi, Dr. Kouji Kobiyama, Dr. Nao Jounai, for providing invaluable advice and technical help in the project. I would also like to thank Dr. Shandar Ahmad for his help in the miRNA bioinformatics analysis. I would like to express my sincere thanks to past and present lab members, both in Singapore and Japan, for the provided assistance and built friendships throughout my PhD course. Thank you all for your affable company in the lab, and for keeping me sane these four years. To friends and people whom I did not manage to mention who have helped in one way or another, thank you. Finally, I would like to give a big thank you to my family for your unwavering support and belief in me.

Table of Contents

Declaration ..... **ii**

Publications ..... **iii**

Acknowledgements ..... **iv**

Summary ..... **viii**

List of Abbreviations ..... **x**

List of Figures ..... **xiv**

Chapter 1: Introduction **1**

1.1 Cancer and DNA damage **3**

1.2 Genomic instability **4**

1.2.1 Sources of DNA damage and genomic instability 4

1.2.2 Double-stranded and single-stranded DNA 5

1.2.3 RNA:DNA hybrids 6

1.3 DNA damage response **10**

1.4 DNA repair **12**

1.5 DAMPs and PAMPs **14**

1.5.1 Nucleic acid as a DAMP 18

1.6 The RNA Polymerase (POL) III family **21**

1.6.1 RNA POL III activity 22

1.6.2 RNA POL III in immune sensing 25

1.6.3 RNA POL III in cancer 25

1.6.4 Clinical trials using RNA POL III inhibitors 26

1.7 MicroRNAs (miRNAs) **27**

1.7.1 Biogenesis 27

1.7.2 MiRNAs in DNA damage 29

1.7.3 MiRNAs in cancer 30

1.8 Eμ-*Myc* mouse model **31**

1.9 Aims **33**

Chapter 2: Materials and Methods **35**

2.1 Mice and cells **36**

2.2 Reagents **36**

2.3 Immunocytochemistry **37**

2.4 Electron microscopy **37**

2.5 ELISA **38**

2.6 Immunoblot **38**

2.7 Immunoprecipitation and mass spectrometry **38**

2.8 Transfection **39**

2.9 MiRNA microarray analysis **39**

2.10 Quantitative PCR **40**

2.11 Flow cytometry **41**

2.12 Statistical Analysis **41**

Chapter 3: Presence of DNA in the Cytosol in response to DNA damage **42**

3.1 Presence of cytosolic DNA in cell lines **43**

3.2 DNA damage increases level of cytosolic DNA **46**

3.3 Accumulation of dsDNA by DNA damage depends on ATM **49**

3.4 Cytosolic DNA does not colocalize with known DNA-binding molecules **51**

3.5 DDX17 expression decreases with DNA damage **55**

3.6 DDX17 expression regulates the presence of cytosolic dsDNA **59**

Chapter 4: RNA:DNA hybrids in cytosolic abundance **61**

4.1 Presence of RNA:DNA hybrids in cytosol **62**

4.2 Specificity of RNA:DNA hybrids **65**

4.3 Inhibition of RNA POL III decreases cytosolic RNA:DNA hybrids **66**

4.4 Presence of RNA:DNA hybrids is independent of DNA damage **70**

4.5 RNA:DNA hybrids are present in human lymphoma tissues **70**

4.6 Cytosolic RNA:DNA hybrids binds components of the miRNA processing machinery 72

Chapter 5: RNA POL III affects miRNA expression but not interferon production..... 75

5.1 RNA POL III regulates the expression of specific miRNAs 76

5.2 Interferon-beta levels are refractory to siRNA against RNA POL III 81

Chapter 6: *Tnfa* deficiency reduces survival of Eμ-*Myc* mice 84

6.1 Decreased survival rate of *Tnfa*-deficient Eμ-*Myc* mice..... 85

6.2 Analysis of *Tnfa*-deficient Eμ-*Myc* mice tumor load 88

Chapter 7: Discussion and future perspectives..... 90

7.1 Summary of key findings 91

7.2 dsDNA and RNA:DNA hybrids are derived from two distinct pathways..... 93

7.3 Detection of nucleic acids by staining 96

7.4 Nature of dsDNA interactions with cytoplasmic proteins..... 99

7.5 RNA POL III-dependent cytosolic RNA:DNA hybrids 101

7.6 Role of DDX17 in DNA damage and miRNA biogenesis 102

7.7 MiRNA expression and RNA:DNA hybrids..... 104

7.8 Innate immune signaling of nucleic acids..... 106

7.9 Tumor progression in Eμ-*Myc* mice..... 108

7.10 Conclusion 111

Bibliography 112

Appendices..... 133

Appendix A. Extract of Mass Spectrometry Mascot Search Results..... 133

Summary

DNA sensors of the innate immune system recognize nucleic acids such as double-stranded DNA (dsDNA) and RNA:DNA hybrids of viral and bacterial origin. These are able to trigger an effective immune response via the production of interferons and cytokines. However, little is known about the existence of cytosolic nucleic acids in virus-free cells such as cancer or transformed cells, and its role in immunosurveillance and tumorigenesis.

In this thesis, we show that cytosolic nucleic acids are present in various non-infected human cell lines. DDX17 is found to bind both dsDNA and RNA:DNA hybrids, exhibiting different activity in accumulation of either nucleic acid. While DDR pathways were shown to regulate dsDNA accumulation, inhibition of RNA polymerase III, but not DNA polymerase abrogated cytosolic RNA:DNA hybrids. Further, as cytosolic RNA:DNA hybrids bind to several components of the microRNA machinery-related proteins, we identified microRNAs that were specifically regulated by RNA polymerase III, providing a potential link between RNA:DNA hybrids and the microRNA machinery.

To investigate the role of cytosolic DNA in tumorigenesis, I crossed Eμ-*Myc* mice with *Tnfa/Tbk1*-double knockout mice, as *Tbk1*-deficient mice are embryonically lethal. To control for the effects of TNF-α, I also crossed *Tnfa*-deficient mice. Mice were observed to have lower survival, due to the dominant role of TNF-α in proinflammatory and cell killing responses. Our data suggest that an efficient immune response is triggered in Eμ-*Myc* mouse model due to intracellular sensing of tumor cells, and possibly cytosolic DNA.

In summary, we have shown the presence of cytosolic dsDNA and RNA:DNA hybrids in virus-free human cells, and revealed possible substrates for sensing in tumor cells to trigger an immune response during tumor progression, as

evidenced by Eμ-*Myc* mice data. Presence of RNA:DNA hybrids were also found to be regulated by RNA polymerase III, which also affects microRNA expression, suggesting a pathway by which hybrids are modulated.

List of Abbreviations

5’ppp	5’ triphosphate
9-1-1	RAD9-RAD1-HUS1
ADP	Adenosine diphosphate
AF488	Alexa Fluor 488
AF555	Alexa Fluor 555
AF549	Alexa Fluor 549
AGS	Aicardi-Goutieres syndrome
AKT	Protein kinase B
ANA	Anti-nuclear antibodies
APC	Allophycocyanin
APH	Aphidicolin
ATCC	American type culture collection BCL1L12 BCL2-like 12
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
BER	Base excision repair
BRCA1	Breast cancer 1, early onset
BSA	Bovine serum albumin
CD	Cluster of differentiation
CHK	Checkpoint kinase
COX IV	Cytochrome C oxidase subunit IV
CXCL10	Chemokine (C-X-C motif) ligand 10
cGAS	Cyclic GMP-AMP
DAI	DNA-dependent activator of interferon-regulatory factors
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DDR	DNA damage response

DDX5	DEAD (Asp-Glu-Ala-Asp) box helicase 5
DDX17	DEAD (Asp-Glu-Ala-Asp) box helicase 17
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAM-1	DNAX accessory molecule-1
DSB	dsDNA breaks
dsDNA	Double-stranded DNA
E2F1	E2F transcription factor 1
EBV	Epstein-Barr virus
E. coli	*Escherichia coli*
FCS	Fetal calf serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
H2AX	H2A histone family, member X
HERV	Human endogenous retroviral
HMGB1	High mobility group box 1
HP1BP3	Heterochromatin protein 1, binding protein 3
hPNPase	Human polynucleotide phosphorylase
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HSV-1	Herpes simplex virus type 1
IL	Interleukin
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
IFN-β	Interferon-beta
Ig	Immunoglobulin
IRES	Internal ribosome entry site
KRBA2	KRAB-A domain containing 2

LINE	Long interspersed nuclear element
LNA	Locked nucleic acid
MCPIP1	MCP-1 treatment-induced protein
miRNA	MicroRNA
MRN	MRE11-RAD50-NBS1
nc	non-coding
ncRNA	Non-coding RNA
NER	Nucleotide excision repair
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	Non-homologous end-joining
NK	Natural killer
NKG2D	Natural killer group 2, member D
NLRP3	Leucine rich repeat and pyrin domain containing[1]
NOS2	Nitric oxide synthase 2, inducible
NUP153	Nucleoporin 153kDa
Oncomir	Oncogenic miRNA
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
POLR3G	Polymerase (RNA) III (DNA directed) polypeptide G (32kD)
Poly A: U	Polyadenylic: polyuridylic acid
Poly I: C	Polyinosinic: polycytidylic acid
PPR	Pattern recognition receptor
PTEN	Phosphatase and tensin homolog
RISC	RNA-induced silencing complex
RB	Retinoblastoma

RITS	RNA-induced transcriptional silencing
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPA	Replication protein A
rRNA	Ribosomal RNA
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SD	Standard deviation
SEM	Standard error of mean
SINE	Short interspersed nuclear element
SLE	Systemic lupus erythematosus
snRNA	Small nuclear RNA
SSB	Single-stranded breaks
ssDNA	Single-stranded DNA
STING	Stimulator of interferon genes protein
TBK1	TANK-binding kinase 1
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TREX1	Three prime repair exonuclease 1
tRNA	Transfer RNA
WT	Wild type
XPO	Exportin
XRN2	5'-3' Exoribonuclease 2

List of Figures

Figure 1.1 The DNA damage response..... 11

Figure 1.2 DNA repair mechanisms in DNA damage help to preserve genomic stability..... 13

Figure 1.3 Pathway for sensing of nucleic acids..... 17

Figure 1.4 Nucleic acids are sensed by intracellular cytosolic sensors 21

Figure 1.5 RNA POL III transcripts and roles in cellular processes..... 25

Figure 1.6 Biogenesis of microRNA and its regulation by interacting factors... 29

Figure 1.7 Overview of the thesis areas of research. 34

Figure 3.1 Specificity of dsDNA antibodies. 43

Figure 3.2 Presence of cytosolic DNA in various cell lines..... 45

Figure 3.3 Staining of cytosolic dsDNA in MRC-5 cells. 45

Figure 3.4 Cytosolic DNA increased in response to DNA damage..... 48

Figure 3.5 Presence of cytosolic DNA increases after DNA damage with Ara-C. 48

Figure 3.6 Cytosolic dsDNA disappeared after treatment with ATM/ATR inhibitor 50

Figure 3.7 Cytosolic DNA partially colocalizes with DNA sensor molecules 52

Figure 3.8 Cytosolic dsDNA does not colocalize with histone proteins..... 53

Figure 3.9 dsDNA does not colocalize with major compartmental proteins..... 55

Figure 3.10 DDX17 potentially binds cytosolic RNA:DNA hybrids. 56

Figure 3.11 DDX17 is partially localized to the cytosol in response to DNA damage. 58

Figure 3.12 *DDX17* knockdown increased the levels of cytosolic DNA 60

Figure 4.1 Presence of RNA:DNA hybrids in the cytosol of human lung cancer cells 63

Figure 4.2 Presence of cytosolic RNA:DNA hybrids in human tumor cell lines 64

Figure 4.3 Specificity of S9.6 antibody for RNA:DNA hybrids. 65

Figure 4.4 Presence of RNA:DNA hybrid does not depend on the DDR. 66

Figure 4.5 The presence of cytosolic RNA:DNA hybrids depends on RNA POL III. 67

Figure 4.6 siPOLR3G abrogated the presence of cytosolic RNA:DNA hybrids. 69

Figure 4.7. Levels of cytosolic RNA:DNA hybrids were not modulated by genotoxic replication inhibitors 71

Figure 4.8 RNA:DNA hybrids are present in human lymphoma tissues 72

Figure 4.9 Immunoprecipitation of cytosolic RNA:DNA hybrids 73

Figure 4.10 Cytosolic RNA:DNA hybrids interact with proteins of the microRNA machinery 74

Figure 5.1 Dysregulation of microRNAs levels in response to RNA POL III inhibition. 78

Figure 5.2 RNA transport and mRNA surveillance pathways are potentially regulated by RNA POL III modulated miRNAs 80

Figure 5.3 RNA POL III inhibition decreased mRNA expression of POL III-regulated miRNA targets 80

Figure 5.4 siPOLR3G does not regulate IFNb levels. 82

Figure 5.5 Interferon response was not affected at low levels of chemical RNA POL III inhibition 83

Figure 6.1 Survival curve of Eμ-*Myc* ;*Tnfa/Tbk1* double-knockout and Eμ-*Myc*;*Tnfa* mice 87

Figure 6.2 Tumor load analysis of Eμ-*Myc*;*Tnfa/Tbk1* double-knockout, Eμ-*Myc*;*Tnfa*, and Eμ-*Myc* mice 89

Figure 7.1 Proposed model of RNA:DNA hybrids and RNA POL III 92

Chapter 1: Introduction

1. Introduction

Recognition of foreign DNA in the cytoplasm or organelles of a cell triggers a DNA-sensing immune response, where the DNA of either viral or bacterial origin activates receptors that signal through pathways to produce interferons and cytokines, and evoke an effective immune response. This mechanism serves to prevent the host cell from infections; however inappropriate activation by host DNA could damage the host cells leading to autoimmunity.

In recent years, cancer immunotherapy has become a viable option as therapeutic strategies harness the immune system to target cancer cells. Previous research within the lab has shown an upregulation of NKG2D ligands (NKG2DL) in response to DNA damage, and that these processes are constitutively active in cancer cells (Lam *et al.*, 2014a). NKG2D ligands are recognized by receptors found on natural killer (NK) cells that hone in to tumor cells for efficient immunosurveillance. In addition to NKG2D, we have also discovered the presence of cytosolic DNA in DNA-damaged murine tumors (Lam *et al.*, 2014a), which may act as a substrate for DNA-sensing in tumor regression. However, the presence of cytosolic nucleic acids in human cells has yet to be investigated in detail.

While DNA damage is involved in tumorigenesis, less is known about the coupled production of aberrant nucleic acids (dsDNA and RNA:DNA hybrids) via compromised genomic integrity. Dysregulation of these components may contribute to the accumulation of DNA damage, eventually leading to tumors. This study thus examines the presence of the cytosolic nucleic acids in cancer cell lines and investigates the regulation of these nucleic acids.

1.1 Cancer and DNA damage

Cancer evolves as a form of rapidly mutating and proliferating mass within the human body. Worldwide, cancer results in millions of death per year and is one of the leading causes of deaths in developed countries (Cancer, 2014). DNA damage is thought to contribute to tumorigenesis, due to high frequency of mutations within the genome. High replication rates of cancer cells would allow these cells to accumulate further errors that affect genetic encoding of information. Hence, cell cycle disruption is one of the most common ways by which oncogenetic mutations drive cells towards dysregulated proliferation, as reviewed by Williams and Stoeber (Williams and Stoeber, 2012).

DNA is exposed to damage every day; sunlight contains ultraviolet rays that can cause up to 1 x 10^5 DNA lesions in a cell per day (Hoeijmakers, 2009). Exposure to exogenous carcinogenic chemicals also introduces DNA damage and mutates the genome. Compromised DNA integrity leads to accumulated mutations, resulting in genetic and epigenetic changes that lead to cancer, which will be discussed below. Damaged tissue also releases associated molecules that trigger inflammation as an immune response in the body (Medzhitov, 2008). While an acute phase of immune response leads to rapid clearance of damaged cells, long-term inflammation may lead to cancer due to continuous release of toxic reactive oxygen species (ROS) (Jaiswal *et al.*, 2000).

Hallmarks of cancer include sustained proliferation, inhibition of growth suppressors, invasion and metastasis, replicative immortality, angiogenesis induction, and resistance of cell death, as reviewed by Hanahan and Weinberg (Hanahan and Weinberg, 2011). Upstream of these processes, genomic instability and activation of cellular components and processes that regulate cells towards tumorigenesis deserve extensive study. Understanding immune sensing against DNA damage would also provide strategies to enhance specific anti-tumor immune responses.

1.2 Genomic instability

Cell proliferation requires error-free replication to ensure high fidelity of genomic information. Cell cycle checkpoints and repair mechanisms during replication facilitate this process. On the other hand, cancer cells are prone to genomic instability due to high frequency of mutations within the genome of a cellular lineage that remain un-repaired. This genomic instability may result from cells being constantly challenged by both exogenous (UV, X- gamma-rays, viruses, DNA intercalating agents such as ethidium bromide) and endogenous (replication errors, ROS produced from normal metabolic by-products such as oxidative deamination) agents that cause DNA damage. Considering that the body is exposed to over thousands of DNA lesions in a day (Lindahl and Barnes, 2000), this contributes to the oncogenetic process as various regions of the genome are deleted or translocated, resulting in fusion genes, oncogene activation, or tumor suppressor gene (TSG) dysregulation, ultimately leading to cell transformation and tumors.

1.2.1 Sources of DNA damage and genomic instability

Replication stress

The activation of oncogenes increases the proliferation rate of cells and deregulates the timing of replication. The high proliferative rate contributes to the increased accumulation of mutations, as the cell cannot repair damage before the next cell cycle proceeds. This phenomenon, also called replicative stress, directly affects the polymerase activity within cells, as reviewed by Aguilera and Gomez-Gonzalez (Aguilera and Gomez-Gonzalez, 2008). This results in the replication fork collapsing due to extended stalling or checkpoint inactivation. Genetic studies in yeast have also shown an importance of DNA ligase I and DNA polymerases for the accumulation of spontaneous DNA recombination breaks (Aguilera and Gomez-Gonzalez, 2008).

Microsatellite repeats

Another contributing factor to DNA breaks occurs with the transcription of microsatellite repeats, which allow for reading slippage in retroelements such as long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs). Particularly in DNA trinucleotide repeats that are termed as fragile sites, these ubiquitous sites of replication may adopt secondary structures that affect replication (Burrow *et al.*, 2010; Franchitto and Pichierri, 2011). Uncommon structures such as hairpins or DNA triplexes result in disassembly of the replisome, a protein complex that assembles on the DNA to carry out DNA replication (Gacy *et al.*, 1995; Bacolla *et al.*, 2006; Chen *et al.*, 2010). These unique structural DNA signatures (hairpins and DNA triplexes) result in genetic instability, which are able to affect immune response in prostate and colorectal cancer (Egawa *et al.*, 1995; Banerjea *et al.*, 2004).

Retroelements (non-coding DNA)

Retroelements and transposons integrate into the genome of cells, and during duplication of these elements, reintegration into target sites might cause insertions and DNA breaks, associated with increased genomic instability (Belgnaoui *et al.*, 2006; Hedges and Deininger, 2007). Further, replication of retroelements like LINEs are prone to slippage via reverse transcription, and RNA:DNA hybrids accumulate if RNase H2 is not effective in degradation (Volkman and Stetson, 2014), which might contribute to DNA damage if RNA:DNA hybrids are not effectively removed. LINE-1 was also shown to induce DNA damage in cancer cells (Belgnaoui *et al.*, 2006)

1.2.2 Double-stranded and single-stranded DNA

The most common DNA breaks may result in free dsDNA or ssDNA depending on the type of damage caused in the genome. Single-stranded DNA breaks

(SSB) occur frequently as DNA lesions, due to oxidative stress by ROS that accumulate in normal cell metabolism (Caldecott, 2008). Replication forks that encounter unrepaired DNA lesions may result in replication blockage or collapse, leading to the formation of dsDNA breaks (DSB) (Kuzminov, 2001). Bacterial or viral DNA is also present in the cytoplasm in infected cells (Fredlund and Enninga, 2014). Tumorigenesis is associated with prolonged DNA damage, which in turn is most commonly associated with DNA breaks, and in particular with DSB. Self-dsDNA is capable of activating innate immune signaling, which is discussed further below in section 1.5.1 'Nucleic acid as a DAMP'. As such, the study of endogenous dsDNA is important in the context of cancer.

1.2.3 RNA:DNA hybrids

The presence of nuclear RNA:DNA hybrids is associated with the occurrence of DNA breaks and transcription (Komissarova *et al.*, 2002; Hamperl and Cimprich, 2014). RNA:DNA hybrids are shown to be involved with genomic instability via Rad51p and homologous recombination (Wahba *et al.*, 2011; Biffi *et al.*, 2013; Wahba *et al.*, 2013). RNA:DNA hybrid formation in yeast by non-coding RNA has been shown to be able to bind to chromatin in a RNAi-machinery dependent manner (Nakama *et al.*, 2012). Interestingly, the bacterial ortholog of Rad51p is RecA, an exchange protein that also promotes RNA:DNA hybrid formation via DNA repair (Kasahara *et al.*, 2000). RecA is also known to bind dsDNA and facilitate ssDNA cross-exchange, likely important for recombination (Zaitsev and Kowalczykowski, 2000). Thus RNA:DNA hybrid formation is associated with replication forks, and disruption of the complex might lead to release of hybrids along with genomic instability.

While many studies mentioned above have focused on nuclear generation of RNA:DNA hybrids, the presence of cytosolic RNA:DNA hybrids is limited to

replication intermediates in virally-infected cells. It is unknown whether endogenous RNA:DNA hybrids are stably present in the cytosol of cells. Hence, the presence of RNA:DNA hybrids and its association with genomic instability poses questions for subsequent cellular activity and how it might relate to diseases that are associated with genomic instability, such as cancer. Here we list circumstances under which RNA:DNA hybrids can occur.

Replication intermediates

DNA replication occurs during the S phase of a cell cycle at replication forks, where DNA is separated into two strands called the leading and lagging strand, which act as templates for replication. Short RNA primers (9 or 10 nucleotides in length) are synthesized by primase and bind the DNA to initiate DNA polymerase downstream elongation of nascent DNA (Harrington and Perrino, 1995). These short RNA:DNA hybrids thus occur at replication sites, with accumulation of multiple RNA:DNA hybrids particularly at the lagging strand, due to the directionality of DNA that requires discontinuous replication. Replication fragments on the lagging strand are termed Okazaki fragments, and when the DNA polymerase collides with another downstream Okazaki fragment, 5' end of the primer is eventually replaced with corresponding deoxyribonucleotides, as reviewed by (Burgers, 2009).

DNA repair mechanisms were also found to associate with RNA:DNA hybrid formation. One example is Rad51p, which repairs DNA breaks in homologous recombination, and was found to regulate accumulation of RNA:DNA hybrids in yeast cells (Wahba *et al.*, 2013).

Transcription and R-loops

Short 8 base-pair RNA:DNA hybrids form in RNA POL II mediated transcription (Komissarova and Kashlev, 1998; Kireeva *et al.*, 2000). RNA:DNA hybrids also form within the RNA POL III complex, where shortening of the hybrid

via destabilization is a cue for transcription termination (Iben *et al.*, 2011). Longer RNA:DNA hybrids, called R-loops can also form during stalled transcription. An R-loop consists of a ssRNA transcript hybridizing to duplex DNA in transcription bubbles of RNA polymerases, and it can occur during stalling of transcription or during replication of mitochondria DNA (Aguilera and Garcia-Muse, 2012). R-loops are formed with the transcribed strand looping to form a RNA:DNA hybrid, though the non-transcribed strand is generally more sensitive to mutations (Beletskii and Bhagwat, 1996). Strikingly, R-loops are also associated with genomic instability during transcription for mRNA biogenesis, as reviewed by Hamperl and Cimprich (Hamperl and Cimprich, 2014), suggesting that the presence of RNA:DNA hybrids may occur in tandem with SSB or DSB.

R-loops formation containing RNA:DNA hybrids are associated with ssDNA regions, and occur at regions of CpG islands, indicative of its GC skew and gene promoter regions (Ginno *et al.*, 2012). Interestingly, this occurrence correlated with unmethylated status of promoters, thus regulating epigenetics within the genome for DNA replication to proceed. In fission yeast, RNA:DNA hybrids were shown to be associated with RNA-induced transcriptional silencing (RITS) complex, and affected heterochromatin formation (Nakama *et al.*, 2012). RNA:DNA hybrids also drive transcriptional silencing by regulating RITS complex formation, which associates with siRNA generation. This intricate interplay of R-loop formation could provide a rapid mechanism by which RNA:DNA hybrid formation would regulate transposon silencing when required.

R-loops are also related to generation of DSB in *Escherichia coli* (*E. coli*) cells and dependent upon ssDNA nicks and starvation (Wimberly *et al.*, 2013). This stress-induced creation of R-loops and by extension, RNA:DNA hybrids, provides another means of aberrant nucleic acid accumulation in the nucleus, which would require an efficient clearance system from nucleases.

G-quadruplexes

RNA:DNA hybrids also occur in G-quadruplexes and are detected in the nuclei of mammalian cells (Biffi *et al.*, 2013; Lam *et al.*, 2013; Xu and Komiyama, 2013). G-quadruplexes are guanine-rich nucleic acid sequences that form a stable four-stranded looping secondary structure through Hoogsteen hydrogen bonding (Burge *et al.*, 2006). Lam et al. 2013, isolated nuclear genomic DNA fragments containing G-quadruplex structures, demonstrating the presence of these structures stably in human genomic DNA (Lam *et al.*, 2013), that can suppress transcription by stopping RNA POL II reads (Eddy and Maizels, 2008).

1.3 DNA damage response

To protect genome integrity, cells first activate the DNA damage response (DDR), which counteracts the adverse consequences of DNA lesions by inducing repair of damaged DNA (Ciccia and Elledge, 2010). Damage that cannot be repaired results in cells undergoing apoptotic cell death or permanent cell cycle arrest.

The DDR pathway triggers two types of sensing and repair mechanisms according to the type of damage that occurs (Fig. 1.1). ssDNA breaks occur with nucleotide misreading or point mutations, and are sensed by replication protein A (RPA) and the RAD9-RAD1-HUS1 (9-1-1) complex, via ATR phosphorylation to downstream CHK1 kinase. Conversely, DSB are sensed by the MRE11-RAD50-NBS1 (MRN) complex, which phosphorylates ATM to activate CHK2 kinase. Signal amplification via ATM/ATR phosphorylation along with the Chk protein kinases allows for many downstream molecules to be activated, notably tumor suppressors such as Breast Cancer 1, Early Onset (BRCA1) and the p53 gene (Ho *et al.*, 2006). Both pathways eventually lead to checkpoint arrest where the genome is assessed for DNA repair. Cyclin-dependent kinase (CDK) activity is also regulated to allow processing of DNA repair before cell cycle continues. Unrepaired damage may cause apoptosis or cellular senescence (Van Nguyen *et al.*, 2007). Hence, the outcome of DNA repair determines if a cell re-enters the cell cycle, or is stalled in senescence if repair cannot proceed.

The DDR also triggers an immune response with the upregulation of IRF1 and IRF3 (Kim *et al.*, 1999; Pamment *et al.*, 2002), as NKG2D ligands activate NK cells in an ATM/ATR dependent manner (Gasser *et al.*, 2005). Release of inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) attract immune cells to the microenvironment, so as to recognize and engulf damaged cells (Leek *et al.*, 1998; Rodier *et al.*, 2009). TNF signaling also activates NF-κB, a master regulator of cell survival (Biton and Ashkenazi, 2011).

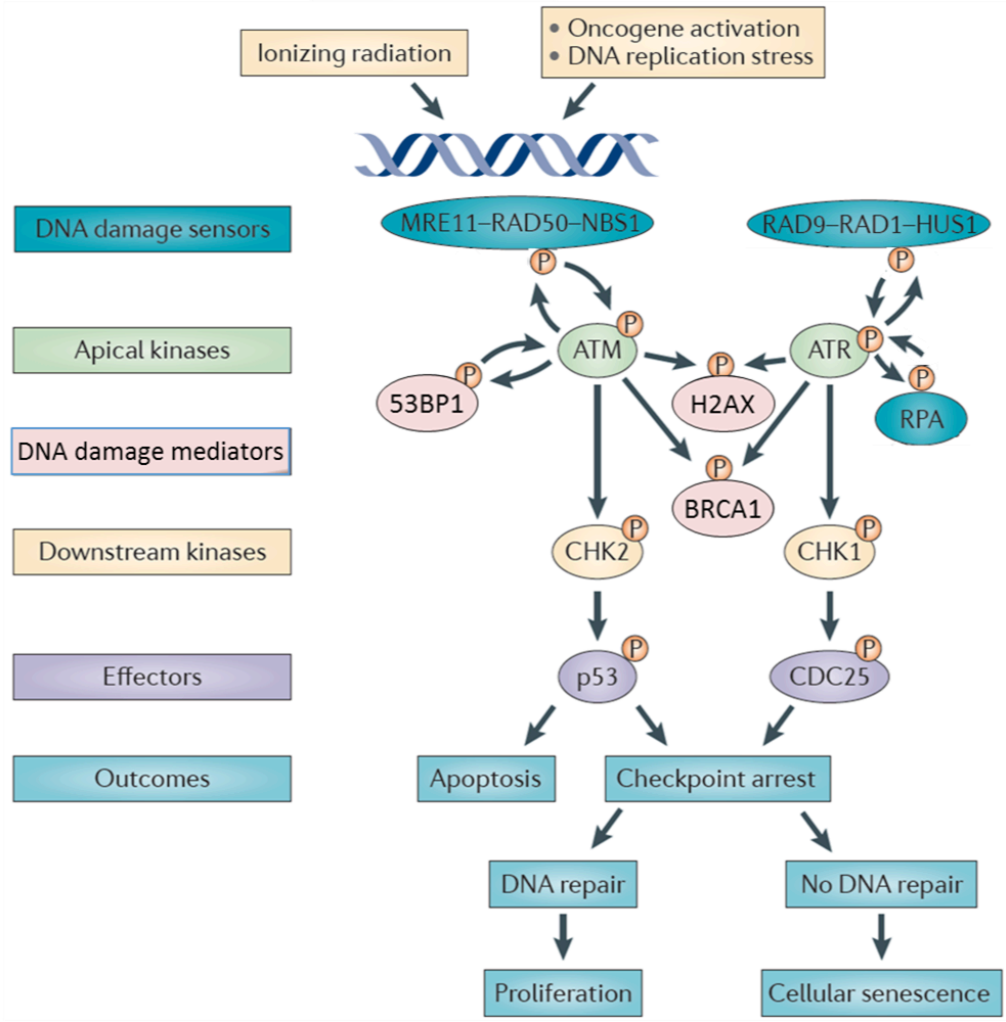


Figure 1.1 The DNA damage response. The DNA damage response (DDR) follows a cascade of signaling steps that are triggered by genotoxic stress. dsDNA or ssDNA breaks in the genome are recognized by the MRE11-RAD50-NBS1 (MRN) complex or Replication protein A (RPA) and RAD9-RAD1-HUS1 (9-1-1) respectively. Downstream kinases ATM or ATR phosphorylate mediators along with important kinases Chk1 and Chk2 that regulate effectors, which in turn affect cell cycle progression, apoptosis, or cellular senescence. Modified with permission from Nature Reviews Cancer publication (Sulli *et al.*, 2012), copyright 2012.

1.4 DNA repair

The DDR activates two distinct pathways to regulate DNA repair (Sulli *et al.*, 2012). Figure 1.2 shows the types of DNA damage that trigger specific pathways of DNA repair. These require key repair proteins, which when mutated would result in formation of tumors. ssDNA repair occurs with mismatch repair (MMR), base excision repair (BER), or nucleotide excision repair (NER). NER recognizes base lesions and repairs it such that the damaged DNA is removed with the excision of a 22-30 base ssDNA (Huang *et al.*, 1992). Conversely, DSB repair is dependent on the stage of cell cycle replication. During most phases of the cell cycle, the cell utilizes non-homologous end-joining (NHEJ), which mediates repair by a sequence-independent complex that comprises of DNA-PK and Ku70, and Ku80 proteins (Spagnolo *et al.*, 2006). This, along with a less characterized microhomology-mediated end-joining (MMEJ), is largely error prone. In contrast, the important S and G2 phases of a cell cycle utilize homologous recombination (HR) that ensures accurate repair via pairing of sister-chromatid templates. HR is usually associated with the MRN complex at the very early stage of cell cycle repair. Rad51 protein accumulates in the nucleus after DNA damage and forms foci, along with BRCA2 to repair DSBs (Tarsounas *et al.*, 2004).

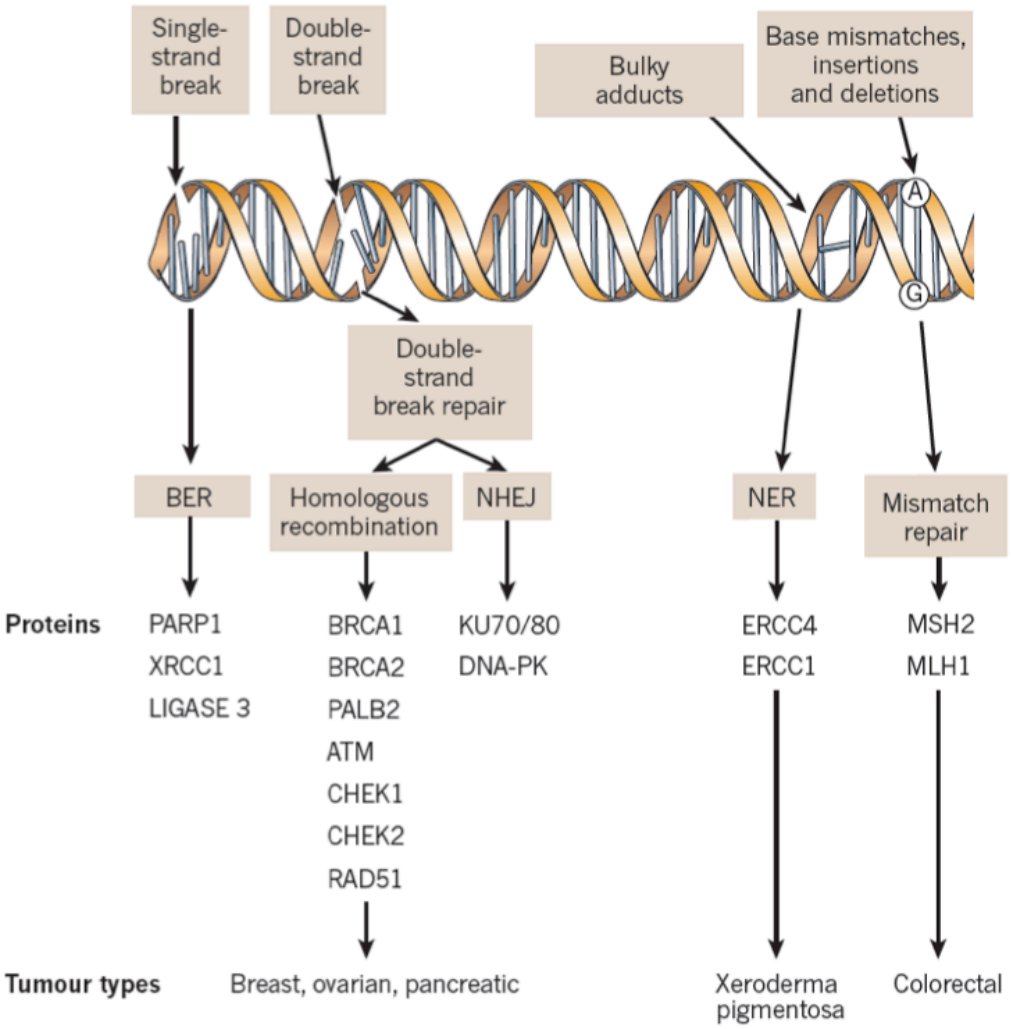


Figure 1.2 DNA repair mechanisms in DNA damage help to preserve genomic stability. The genome is assaulted with different types of DNA damage, and DNA repair mechanisms exist for different types of lesions, where the DDR recruits specific proteins that help to process the genome for repair. Tumor types found to be associated with a defect in each repair pathway are shown. BER, base excision repair; NER, nucleotide excision repair; NHEJ, non-homologous end-joining. Modified with permission from Nature Publishing Group (Lord and Ashworth, 2012), copyright 2012.

1.5 DAMPs and PAMPs

Characteristics of DAMPs and PAMPs

In addition to the DDR, irradiation or genotoxic compounds also trigger the release of danger-associated molecular patterns (DAMPs) or alarmins, as reviewed by Oppenheim and Yang, and Bianchi (Oppenheim and Yang, 2005; Bianchi, 2007). This is in contrast to pathogen-associated molecular patterns (PAMPs) that are non-self antigens released in response to pathogen infection by viruses or bacteria, as reviewed by Mogensen (Mogensen, 2009). DAMPs and PAMPs can be categorized into lipid-related, sugar-related, metabolite-related, nucleic acid-related, and protein related, as reviewed by Jounai and colleagues, and Srikrishna and Freeze (Srikrishna and Freeze, 2009; Jounai *et al.*, 2012). Endogenous DAMP signals may be released from necrotic cells, apoptotic cells, or secreted from activated immune cells during microbial, viral infection, or cellular injury (Srikrishna and Freeze, 2009; Jounai *et al.*, 2012) . Examples of DAMPs include DNA, RNA, ROS (reactive oxygen species) and nucleotides, as well as proteins such as high mobility group box 1 (HMGB1) and S100 (Wang *et al.*, 1999; Boyd *et al.*, 2008; Jounai *et al.*, 2012). These elicit an inflammatory response leading to cell clearance and tissue repair. DAMPs have been shown to play an important role in various infectious and autoimmune diseases (Cunha *et al.*, 2012; Tsai *et al.*, 2014).

Pathways for sensing DAMPs and PAMPs

DAMPs and PAMPs are detected by pattern recognition receptors (PRRs) as part of the host sensing system. As reviewed by Takeuchi and Akira, PRRs may be membrane-bound, or cytosolic in nature (Takeuchi and Akira, 2010). Examples of PRRs include Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), and other cytosolic nucleic acid sensor receptors. Of note, TLR receptors 3, 7, and 9, which mainly sense nucleic acids are primarily localized in the endosome. Activation

of PRRs trigger specialized signaling pathways that result in secretion of type 1 interferon (IFN), or proinflammatory cytokines such as IL-1β and IL-18. These may amplify inflammatory responses via maturation of antigen-presenting cells (APCs) (Li *et al.*, 2004), and activate specific T cell immune responses such as cell cytotoxicity, leading to cell death. Figure 1.3 shows the types of DAMPs and PAMPs, with corresponding intracellular nucleic acid sensing systems that activate the immune system, leading to host defense against pathogen infection and autoimmunity.

Self vs. non-self nucleic acid antigens

A fundamental question regarding nucleic acids that are part of DAMPs and PAMPs is how their PRRs ignore self nucleic acids. In particular, nucleic acid structures comprise of a relatively uncomplicated composition based on four nucleotides, making it difficult to chemically distinguish self and non-self nucleic acids. Thus this may cause erroneous recognition of self-antigens, leading to unwanted inflammation against antigens.

Structural modifications of nucleic acids may be a solution to this conundrum; these are present in viral replication but absent in self-nucleic acids. As an example, 5' triphosphate (5'ppp)-dsRNA is a modified structure found only in replicating RNA viruses, and is sensed by RIG-I to activate IFN-α (Hornung *et al.*, 2006; Schlee *et al.*, 2009). However this is not always foolproof, as the B-form structure of canonical DNA (Watson and Crick, 1953) also induces TLR-independent activation of interferon-beta and NF-κB (Ishii *et al.*, 2006). This suggests that self-activation of PRRs may occur, resulting in autoimmune diseases. In addition, other DNA structures such as Z-DNA and G-quadruplexes also associate with genomic instabilities, reviewed by Zhao and colleagues (Zhao *et al.*, 2010), which might lead to DSB, thus triggering the DDR and the innate immune response (Rodriguez *et al.*, 2012).

Compartmentalization of PRRs also places importance on whether an antigen is sensed by cells. DAMPs are sensed by various sensors located in organelle compartments or in the cytosol, to ensure immediate detection of foreign antigens. Toll like receptors (TLRs) are located on the membranes of such compartments, while others such as NLRP3 and DAI are located in the cytosol. Thus, accumulation of DAMPs in unwarranted organelle compartments might trigger an unwanted immune response, resulting in inflammation or autoimmune diseases.

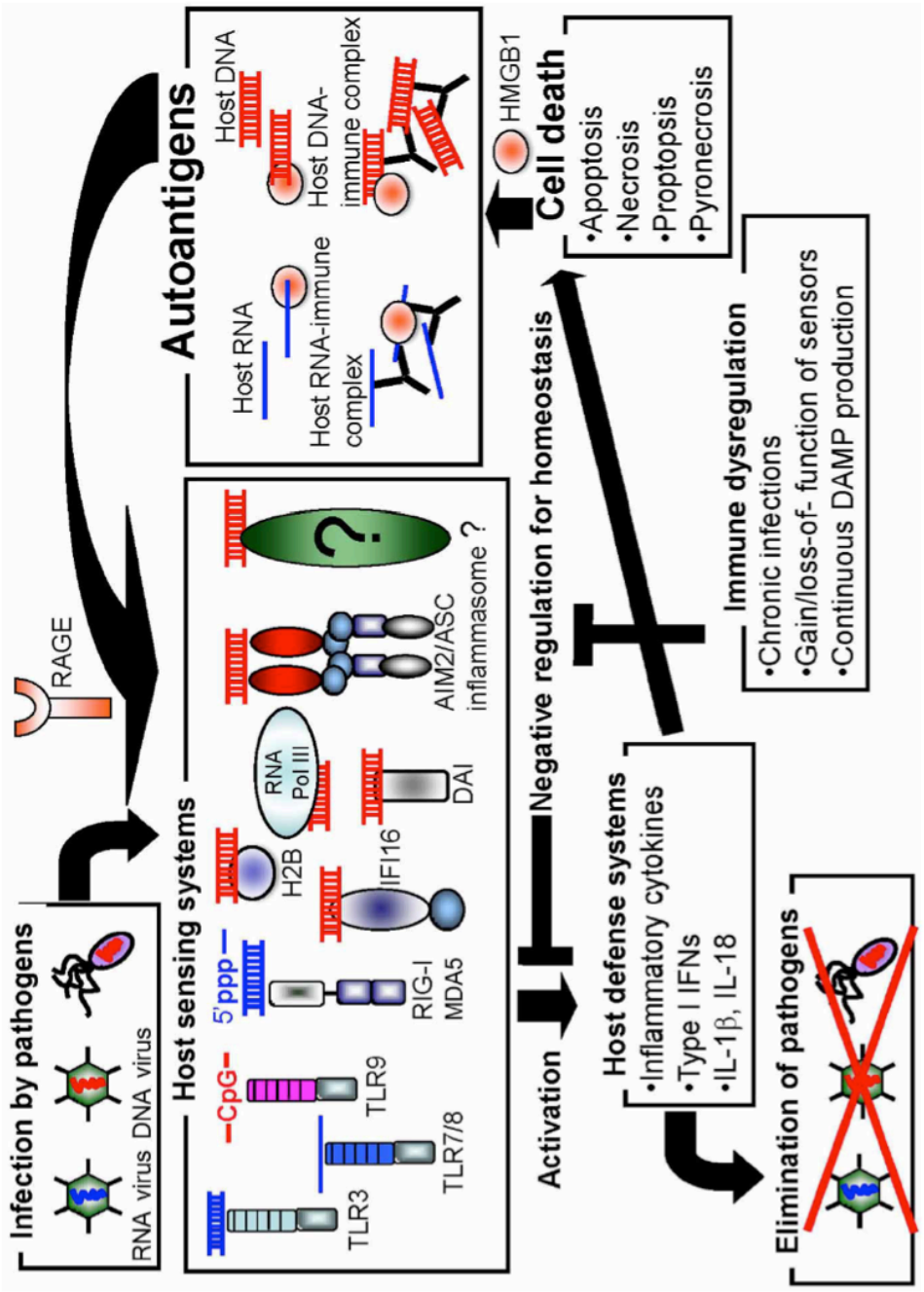


Figure 1.3 Pathway for sensing of nucleic acids.

Figure 1.3 Pathway for sensing of nucleic acids. The host sensing system encompasses various TLRs and cytosolic sensors that detect endogenous autoantigens such as RNA, DNA, or immune complexes, or exogenous antigens from pathogens such as viruses and bacteria. Activation of the host sensors that may locate at endosomes or the cytosol leads to a cascade signaling which results in production of inflammatory cytokines and type 1 IFNs as a host defense system for elimination of pathogens or diseased cells. Immune dysregulation thus disrupts the host defense system leading to pathogen invasion or autoimmunity. Reprinted with permission from Frontiers in Cellular and Infection Microbiology (Jounai *et al.*, 2012) copyright 2012.

1.5.1 Nucleic acid as a DAMP

Accumulation of cytosolic nucleic acids due to genomic instability mechanisms mentioned in the previous section could provide another means by which aberrant DNA is detected in the cytoplasm by DNA sensors, acting specifically as a DAMP in tumor cells. This would provide a means for the immune system to detect cancerous cells via induction of proinflammatory cytokines, chemokines and ligands for activating immune receptors (Gasser and Raulet, 2006a; Gasser and Raulet, 2006b; Weitzman et al., 2004).

dsDNA and ssDNA

A variety of DNA sensors have been discovered in the cytosol, which leads to signaling by STING or MYD88 for interferon and cytokine response. Some evidence has shown that leukemic tumor cells release DNA that is able to disrupt bone marrow and causes apoptosis of surrounding non-tumorigenic cells (Dvorakova *et al.*, 2013). Conversely, cells were found to be non-responsive to ssDNA transfection, since dsDNA but not ssDNA could trigger IFN and IL-8 production in HEK293, suggesting that dsDNA is a more potent immunogen (Ishii *et al.*, 2006).

Evidence of nucleic acid DAMPs in the development of autoimmune disease has been shown in *Dnase1*-deficient mice that develop high titers of anti-nuclear antibodies (ANA) with reactivity to nucleosomes, ssDNA, and dsDNA, suggesting reactivity of ANA to DNA-protein complexes (Napirei *et al.*, 2000). These mice also show characteristics of systemic lupus erythematosus (SLE). Further, SLE patients have been found to have mutations in *DNASE1* (Yasutomo *et al.*, 2001). Interestingly, UV-damaged DNA transfected into mouse dendritic cells (DCs) requires STING but not TLR9 (Gehrke, Immunity, 2013). The pathway by which cell damage activates downstream signaling remains much to be studied.

TREX1 is a DNA-specific exonuclease in mammalian cells (Lindahl *et al.*, 2009). Yang et al. showed that TREX1 relocates to the replication foci in the nucleus after DNA damage (Yang *et al.*, 2007), suggesting its role in DNA repair.

Trex1-deficient mice accumulate ssDNA originating from endogenous retroelement replication, with higher expression of Tnfα, IL1b, Nos2 and cxcl10 in brain tissues (Stetson *et al.*, 2008). High levels of TREX1 are expressed in immune cells, induced by proinflammatory stimuli. Upon UV stimulation, TREX1 translocates from the cytoplasm to the nucleus of cells (Christmann *et al.*, 2010).

RNA:DNA hybrids

RNA:DNA hybrids were first proposed to play a role in immune sensing, when *RnaseH2* mutation leads to Aicardi-Goutieres syndrome (AGS), a neuroinflammatory disorder suggesting the processing of RNA:DNA hybrids are important to prevent inflammatory response (Crow *et al.*, 2006). Additionally, *RnaseH* deficiency in *E. coli* resulted in higher killing ability of macrophages (Crow *et al.*, 2006), suggesting that increased amounts of RNA:DNA hybrids enchance activation of the innate immune system.

Eukaryotic cells have two types of RNase H (1 and 2) that degrade RNA:DNA hybrids. RNase H1 hydrolyzes RNA through its hybrid binding domain, which preferentially binds to RNA:DNA hybrids by 25-fold over dsRNA (Nowotny *et al.*, 2008). RNase H2 is a trimeric complex that hydrolyzes 5'-phosphodiester bonds of the RNA strand of a RNA:DNA hybrid (Rychlik *et al.*, 2010). Replicating retroviruses require RNase H activity, which degrades the ssRNA strand from the nascent dsDNA viral genome during reverse transcription (Wohrl and Moelling, 1990). Both types of *Rnase* deletions are embryonically lethal; *RnaseH1* deletion results in mitochondrial DNA depletion (Cerritelli *et al.*, 2003) while *RnaseH2* deletion results in chromosome instability and elevated DNA damage (Reijns *et al.*, 2012). Notably, RNase H enzymes may also help to suppress R-loop formation. Of

note, the human genome contains large numbers of stable human endogenous retroviral (HERV) insertions, and suppression by RNase H would prevent replication of these transcripts (de Parseval *et al.*, 2003).

Cytosolic RNA:DNA hybrids arising from various sources can be sensed by intracellular cytosolic sensors (Fig. 1.4). Furthermore, immunostimulatory properties have been shown by RNA:DNA hybrids through sensing by TLR9 in murine DCs (Rigby *et al.*, 2014). Following transfection of viral 60 bp RNA:DNA hybrids, IL-6, IFN-α, and TNF-α cytokines were produced by both murine DCs and human peripheral blood mononuclear cells (PBMCs). These responses were found to be MyD88-dependent. Bacterial RNA:DNA hybrids were also found to trigger the NLRP3 inflammasome, with cytosolic hybrids being detected in infected macrophages within an hour of infection (Kailasan Vanaja *et al.*, 2014).

RNA:DNA hybrids from infection by bacteria or viruses

RNA:DNA hybrid formation has been widely known to occur during reverse transcription in retroviral infection (Takano and Hatanaka, 1975a; Horton and Finzel, 1996). RNase H activity degrades the RNA strand of the formed RNA:DNA hybrid after reverse transcription, releasing the nascent DNA to hybridize to the genome. Strikingly, RNA:DNA hybrids are found in endosomal compartments within cells (Rigby *et al.*, 2014), suggesting an engulfment process related to the sensing of these hybrids from viral or bacterial sources. RNA:DNA hybrids are transiently generated in the cytosol of virus-infected cells via reverse-transcription of an viral RNA template strand (Takano and Hatanaka, 1975a, b; Horton and Finzel, 1996; Abbink and Berkhout, 2008). During re-integration of retroelements, reverse transcription occurs with the formation of RNA:DNA hybrids, which is thought to be inhibited by TREX1 or RNase H2 (Stetson, 2012). Increased RNA:DNA hybrids facilitate cytosolic dsDNA accumulation through reverse transcription, which may trigger DNA sensors such as IFI16 and cGAS for sensing via STING.

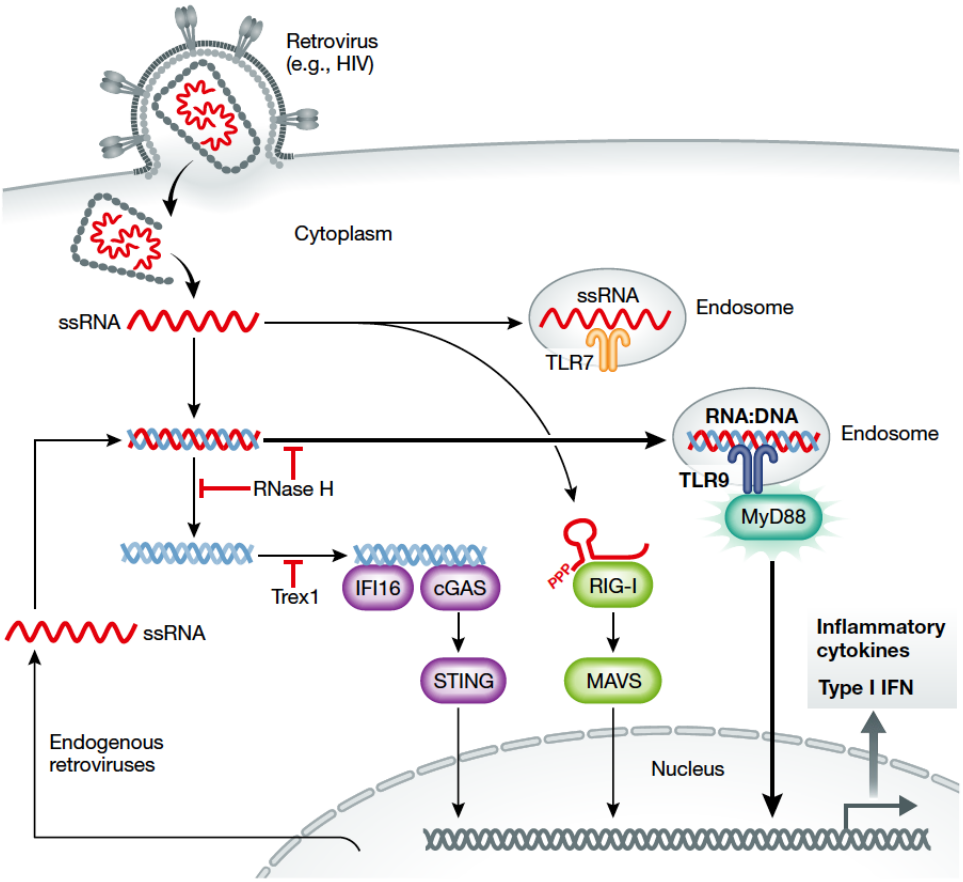


Figure 1.4 Nucleic acids are sensed by intracellular cytosolic sensors. While ssRNA in the cytosol are sensed by TLR7 in the endosome, or RIG-1 in the cytosol, ssRNA from endogenous retrovirus or retrovirus in the cytosol can form RNA:DNA hybrids. RNA:DNA hybrids are normally degraded by RNase H. However, when RNA:DNA hybrids accumulate in the endosome, they are recognized by TLR9/MyD88 signaling pathway. RNA:DNA hybrids can also be reverse transcribed into dsDNA which can be sensed by DNA sensors IFI16, and cGAS. Reprinted with permission from EMBO (Jensen and Paludan, 2014) copyright 2014.

1.6 The RNA Polymerase (POL) III family

RNA polymerases are known to transcribe a nascent ssRNA from double stranded DNA. Most notably, RNA POL II is required for the transcription of mRNAs in genome duplication and cell division (Yonaha *et al.*, 1995). The unique interaction of RNA within unwound dsDNA provides the formation of a temporary RNA:DNA hybrid. However in recent years, RNA POL III has rose in prominence as a cytosolic DNA sensor that recognizes synthetic A-T rich DNA (Ablasser *et al.*, 2009; Chiu *et al.*, 2009). It then reverse transcribes DNA via the formation of

RNA:DNA hybrids, to produce dsRNA that triggers RIG-I to induce an interferon response. In particular, dysregulation of RNA POL III is also associated with cancers (Winter *et al.*, 2000; Moinzadeh *et al.*, 2014). Given the role of RNA POL III in the formation of nucleic acids such as RNA:DNA hybrids that were previously shown to be immunogenic (Fig. 1.4), we chose to focus on RNA POL III and its functional role in immune sensing and cancer.

1.6.1 RNA POL III activity

RNA POL III is the largest RNA polymerase that contains 17 subunits, including a DNA binding site, functioning to catalyze the transcription of nuclear DNA into RNA (Dieci *et al.*, 2007; Lorenzen *et al.*, 2007; Dieci *et al.*, 2013). In contrast to POL I, which is essential for the transcription of ribosomal RNA, and POL II, which is required for a wide variety of transcripts including protein-coding mRNAs and microRNAs (miRNA), POL III-RNA transcripts are characterized by their non-coding infrastructural functionality (Sentenac, 1985). Forms of this enzyme were purported to localize within different cellular regions; III_B is detected in the cytosol, while III_A is found mainly in the nucleus. While it is found both in the nucleus and cytoplasm, two-thirds of RNA POL III activity was in the cytoplasm (Jaehning and Roeder, 1977).

POL III-driven RNA transcripts have roles in multiple cellular processes (Fig. 1.5). The most fundamental role of POL III transcription involves providing a supply of tRNAs required for the translation of mRNA read by ribosome to produce a nascent polypeptide (Weinmann and Roeder, 1974). POL III also transcribes for 5S rRNA, which is required in the assembly of a functional ribosome. While majority of the above-mentioned RNA POL III transcripts are involved in protein synthesis, a portion of RNA POL III transcripts that are non-coding (nc) have been studied for their role in regulating genetic activity to affect cellular processes (Dieci *et al.*, 2013).

RNA POL III-driven ncRNAs include U6 small nuclear RNAs (snRNAs) that assemble in splicesosome (Kambach *et al.*, 1999) to assist in cleavage of introns in genomic sequences to form a functional mRNA for translation, and short interspersed nuclear element (SINE)-encoded RNAs which are widely found in the human genome (Kramerov and Vassetzky, 2005; Dieci *et al.*, 2013). Interestingly SINEs are upregulated in response to DNA damaging agents (Hagan and Rudin, 2007), signifying its role in DNA breakage and DDR. In particular, Alu RNAs, a subset of SINEs can downregulate protein expression as anti-sense inhibitors of mRNAs maturation or translation (Pagano *et al.*, 2007).

Human RNA POL III is also able to transcribe microRNAs (miRNAs), as regions of POL III-occupied miRNAs were identified (Ozsolak *et al.*, 2008). MiRNA-coding sequences were also found within POL III transcribed Alu repeats of the human genome, suggesting the connection between repetitive elements, POL III, and miRNA biogenesis and regulation (Borchert *et al.*, 2006).

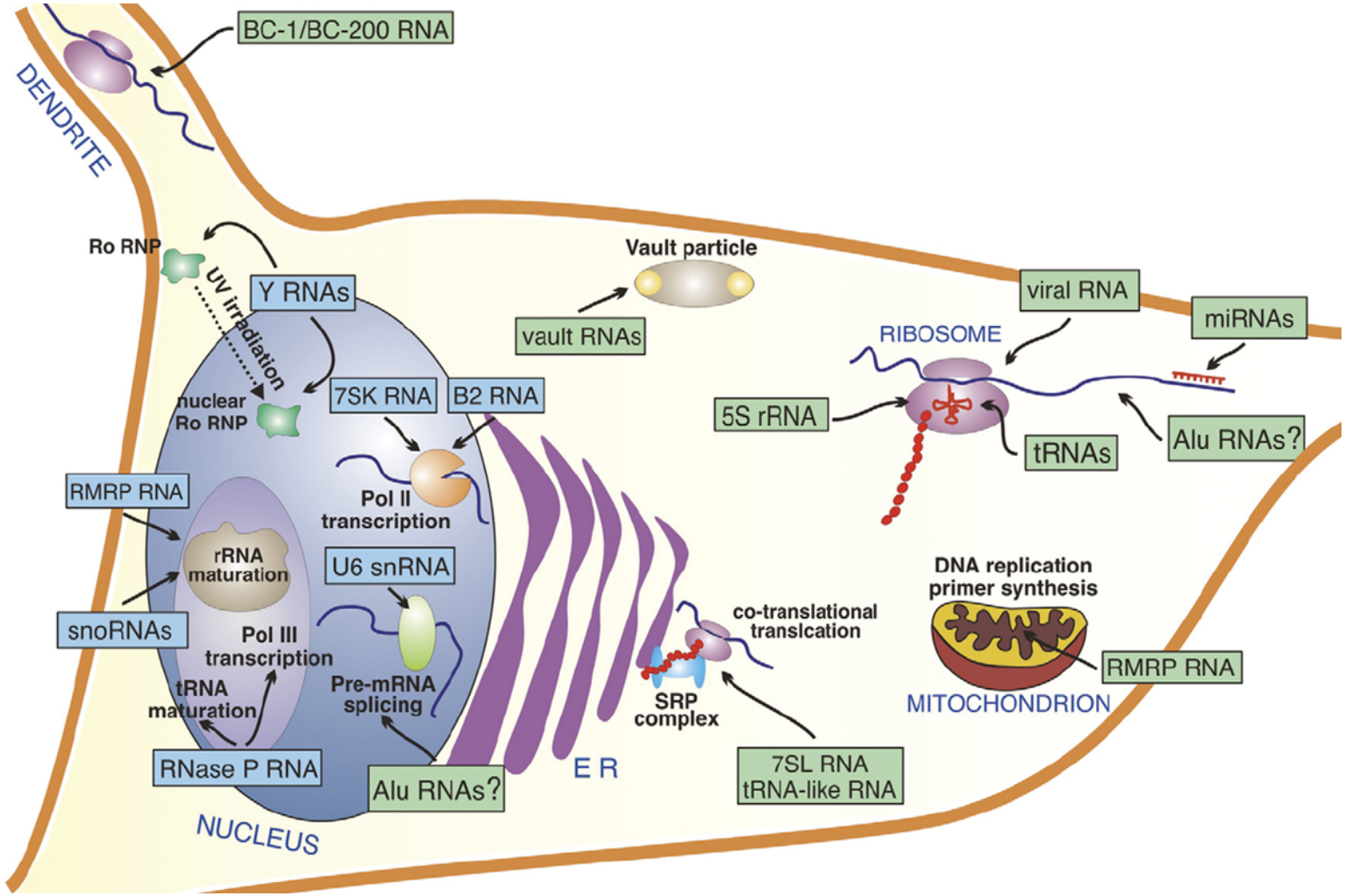

BC-1/BC-200 RNA
DENDRITE
Ro RNP
UV irradiation
Y RNAs
nuclear
Ro RNP
7SK RNA
B2 RNA
RMRP RNA
Pol II
transcription
rRNA
maturation
U6 snRNA
snoRNAs
Pol III
transcription
tRNA
maturation
Pre-mRNA
splicing
RNase P RNA
Alu RNAs?
NUCLEUS
Vault particle
vault RNAs
E R
co-translational
translcation
SRP
complex
7SL RNA
tRNA-like RNA
viral RNA
miRNAs
RIBOSOME
5S rRNA
tRNAs
Alu RNAs?
DNA replication
primer synthesis
RMRP RNA
MITOCHONDRION

Figure 1.5 RNA POL III transcripts and roles in cellular processes. Various types of non-coding RNAs transcribed by RNA POL III act in the nucleus (Blue boxes) and cytoplasm (Green boxes) to regulate cellular processes. Alu transcripts have poorly defined functions and are indicated with a question mark. Modified with permission from ScienceDirect: Trends in Genetics (Dieci *et al.*, 2007) copyright 2007.

1.6.2 RNA POL III in immune sensing

Cells transformed by simian virus 40 (SV40) were shown to upregulate POL III transcription components (Valentine and Smith, 2010). More recently, RNA POL III was also found to transcribe synthetic AT-rich DNA and Epstein-Barr virus (EBV)-encoded RNAs in the cytosol, a distinct feature from its original nuclear genome transcription (Ablasser *et al.*, 2009; Chiu *et al.*, 2009). RNA POL III transcribes the dsDNA into ssRNA containing 5'-triphosphate (5'-ppp), which is recognized via RIG-1 and signaled through the TBK1/IRF3 pathway to produce IFN-β. RNA POL III inhibition also blocked IFN-β production in response to infection with bacterium *Legionella pneumophila*, viruses herpes simplex virus type 1 (HSV-1), and Epstein Barr virus (EBV) (Ablasser *et al.*, 2009; Chiu *et al.*, 2009). Vaccinia virus E3 protein was further shown to inhibit this RNA POL-III dsDNA-sensing pathway (Valentine and Smith, 2010), suggesting the significance of this pathway in the recognition of viruses for killing of infected cells.

1.6.3 RNA POL III in cancer

RNA POL III transcribes tRNAs required for protein synthesis and ncRNAs that can modulate gene expression for cell processes (Kunkel *et al.*, 1986; Canella *et al.*, 2010). POL III was also implicated in DNA sensing and the induction of IFN-β (Ablasser *et al.*, 2009; Chiu *et al.*, 2009). Given its role in modulating oncogenes and tumor suppressor genes, and immune sensing, POL III activity was consequently found to be associated with cancer progression. POL III transcripts were initially found to be elevated in transformed cells (Singh *et al.*, 1985; Carey *et al.*, 1986); POL

III-driven transcripts BC1 and BC200 were only detectable in tumor cells while normal neighboring tissues did not express these transcripts (Chen *et al.*, 1997a; Chen *et al.*, 1997b). Mice with melanomas had hyperactive POL I and III, while POL II levels remained normal (Schwartz *et al.*, 1974). Overexpression of RNA POL III was found in human ovarian carcinoma (Winter *et al.*, 2000). RNA POL III was found to interact with many proteins involved in cell proliferation and known tumor regulating proteins, e.g. retinoblastoma (RB) (Gjidoda and Henry, 2013) and BRCA1 (Veras *et al.*, 2009). Decreased RNA POL III transcription factor Brf1 expression increased anchorage-independent cell proliferation, a characteristic of cancer cells, alongside a decrease in RNA POL III transcription (Johnson *et al.*, 2008). On the other hand, tumor suppressor PTEN, which is commonly mutated in cancers, represses RNA POL III transcription (Woiwode *et al.*, 2008). RNA POL III transcripts and associated transcription factors are hence able to target tumor suppressor genes and oncogenes (Marshall and White, 2008) that normally lead to cancers.

1.6.4 Clinical trials using RNA POL III inhibitors

RNA POLs are potential targets in drug design for cancer therapy, due to their nature in transcribing genes essential for cell function, such that dysregulation of these processes result in cancer. For example, clinical trials for cancer therapy are underway by using RNA POL II inhibition to result in cell cycle arrest (Byers *et al.*, 2005). Additionally, RNA POL III may be another potential cancer therapy candidate given its role in cell proliferation and contribution to oncogenesis. For example, RNA POL III antibodies were found to be risk factors for scleroderma renal crisis in systemic sclerosis patients (Hesselstrand *et al.*, 2012).

Drugs have also been manufactured to combat the transcription pathways targeted RNA polymerases. TAS-106 is a RNA polymerase inhibitor of I, II, and III, and was used in a clinical trial of platinum-failure metastatic head and neck cancer or solid malignancies. This was terminated due to insufficient treatment benefits and

complications at high dosage levels, although complementary treatment with other drugs was considered for further study (Hammond-Thelin *et al.*, 2012; Tsao *et al.*, 2013). However, Favipiravir (T-705) is a RNA-dependent RNA polymerase inhibitor of influenza viruses. Its active form, favipiravir-ribofuranosyl-50-triphosphate (RTP) acts as a pseudo-purine nucleotide to prevent further incorporation of nucleotides for viral RNA replication (Furuta *et al.*, 2013).

1.7 MicroRNAs (miRNAs)

1.7.1 Biogenesis

MiRNAs are 18-25 nucleotide-long RNAs that post-transcriptionally regulate gene expression by complimentary binding to an mRNA strand to mediate processes such as apoptosis, proliferation, and cell signaling (Kloosterman and Plasterk, 2006; ChunJiao *et al.*, 2014). MiRNAs are located within the genome, and initially transcribed as primary miRNAs (pri-miRNAs) by RNA POL II or III in the nucleus (Lee *et al.*, 2004). This process may be dependent on gene transcription if the miRNA is located within the intron (intronic region) of a host gene, or independent pri-miRNA transcription if the miRNA has its own promoter (intergenic region) (Ozsolak *et al.*, 2008). Formation of a mature miRNA requires a two-step processing that is elaborated in Figure 1.6 that starts with cleavage of the primary miRNA to form a stem loop precursor miRNA (pre-miRNA) by the DROSHA-DGCR8 complex (Han *et al.*, 2004). Many proteins also interact to regulate pri-miRNA processing, including BRCA1 oncogene (Kawai and Amano, 2012), and RNA helicases DEAD (Asp-Glu-Ala-Asp) box helicase 5 and 17 (DDX5/DDX17) (Fukuda et al., 2007). Subsequent export to the cytoplasm is mediated by exportin 5 (XPO5) with Ran-GTP, and a second-step processing by Dicer-TRBP complex that cleaves the hairpin loop to release a miRNA duplex (Yi *et al.*, 2003; Han *et al.*, 2004). Further unwinding and loading of the mature single-stranded miRNA into the RNA-induced

silencing complex (RISC) allows it to function in RNAi gene silencing, targeting its complementary mRNA. Degradation of pre-miRNAs is regulated by MCPIP1 (Suzuki *et al.*, 2011), XRN2 (Chatterjee and Grosshans, 2009) or hPNPase (Das *et al.*, 2010).

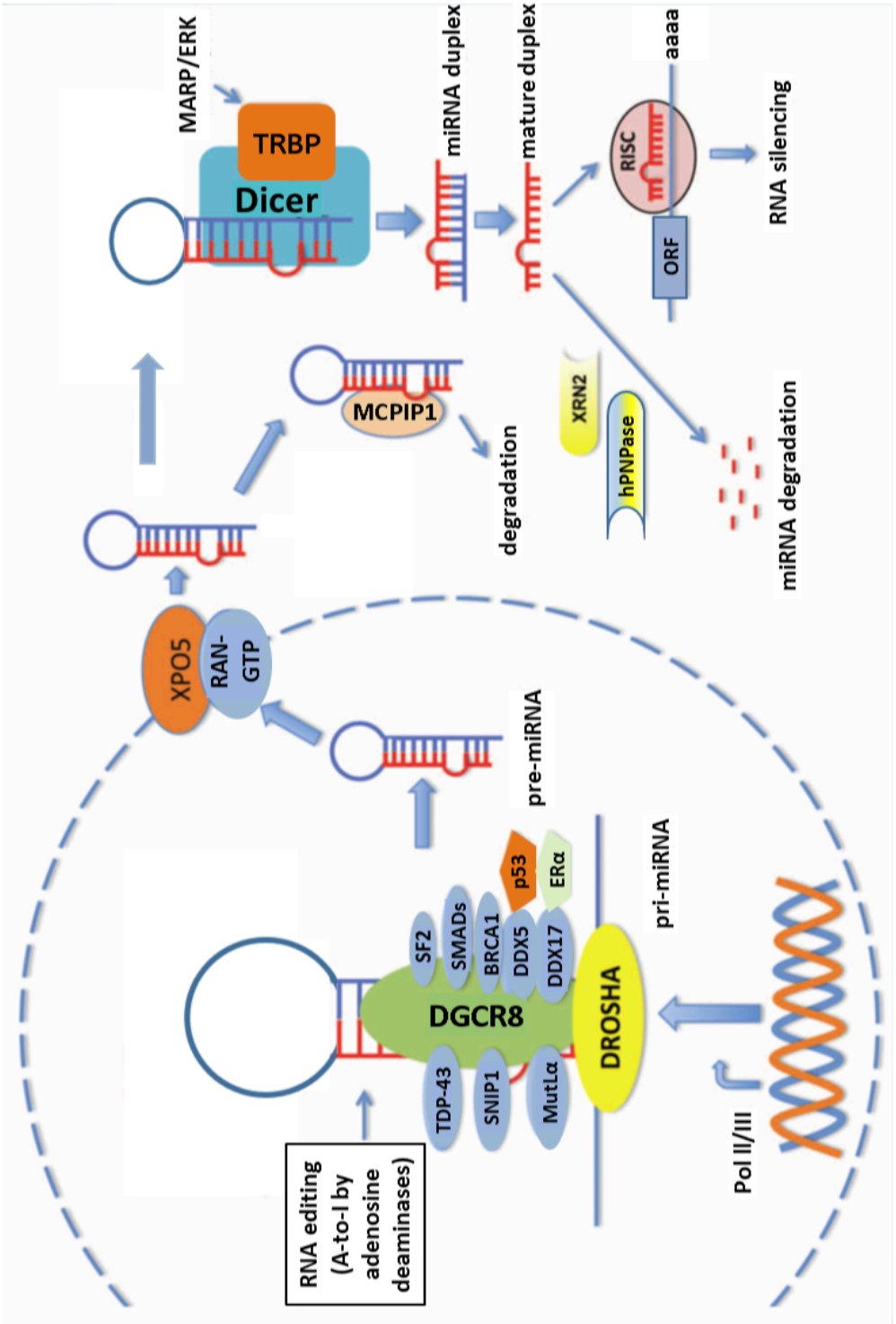


Figure 1.6 Biogenesis of microRNA and its regulation by interacting factors. MiRNAs are transcribed from the intronic or intergenic portions of the genome by RNA POLs II or III (POL II/III) into pri-miRNAs. The DROSHA-DGCR8 complex binds and further processes the miRNA by cleaving to release the stem loop, thus generating a pre-miRNA, which is exported via XPO5-containing nuclear export receptor-complex into the cytosol. The cytosolic Dicer-TRBP complex then binds and removes the hairpin loop to produce a miRNA duplex, of which one strand of the duplex will be a mature miRNA and loaded into the RISC complex to execute gene silencing against a transcript complementary to its sequence. MiRNA degradation is regulated by XRN2 and hPNPase that digest single-stranded miRNAs, and MCPIP1 that removes unprocessed pre-miRNAs. Modified with permission from Landes Bioscience: Cell Cycle (Wang and Taniguchi, 2013), copyright 2013.

1.7.2 MiRNAs in DNA damage

MiRNA biogenesis is affected by DNA damage, which is to be expected since miRNAs regulate expression of many genes, including DNA damage and repair genes. Transcription of miRNA can be regulated in response to DNA damage. P53 is a tumor suppressor that pauses the cell cycle, activates DNA repair proteins, and directly targets miR-34a for upregulation, providing a positive feedback loop to increase tumorigenesis (Okada *et al.*, 2014). MiR-192 and miR-215 are also dependent on p53 and are upregulated in response to genotoxic stress (Braun *et al.*, 2008; Georges *et al.*, 2008). MiRNA export has been shown to be dependent on ATM/ATR, as neocarzinostatin-mediated DNA damage results in XPO5 mediated transport of miRNAs into the cytoplasm (Wan *et al.*, 2013). MiR-138 decreases H2A Histone Family, Member X (H2AX) expression in human cells, and affects genomic stability and DNA repair (Wang *et al.*, 2011). Other pathways that are affected by miRNAs include cell cycle checkpoint, DNA repair, and DDR-dependent pathways (Wang and Taniguchi, 2013). MiRNA is purported to involve both upstream and downstream of DNA damage signaling, as some miRNAs affects DNA damage molecules, while DNA damage also directly affect miRNA transcription. Thus, a potential feedback loop between miRNAs and DNA damage might regulate both signaling pathways in cellular processes.

1.7.3 MiRNAs in cancer

Human microRNA genes were found to locate within putative cancer-associated genomic regions and fragile sites along with breakpoints (Calin *et al.*, 2004). The possibility of these intronic miRNAs to be expressed when cancer-associated genes are transcribed, hints at the role that miRNA might play in regulating genes that affect tumorigenesis. Oncogenic miRNAs (oncomirs) is a group of miRNAs that target mRNAs coding for tumor suppressor genes or oncogenes, thus regulating these genes and their downstream effects on cellular processes associated with cancer, such as apoptosis and proliferation (Macfarlane and Murphy, 2010). For instance the Let-7 family down regulates c-*Myc* mRNA and protein expression to reduce proliferation in Burkitt lymphoma cells (Sampson *et al.*, 2007). A comprehensive list of miRNA involved in various human cancers is shown in Babashah and Soleimani (Babashah and Soleimani, 2011).

Many RNA-binding proteins such as DDX5 and DDX17, that regulate miRNA biogenesis, are also implicated in cancer, signifying an interplay of the miRNA processing machinery and other cellular processes (van Kouwenhove *et al.*, 2011). Strikingly, pre-miRNAs tend to accumulate in tumor cells as compared to normal tissue (Thomson *et al.*, 2006) with the repression of mature miRNA formation (Kumar *et al.*, 2007), allowing for cellular transformation and progression towards tumorigenesis. For instance, XPO5 mutations cause a similar decrease of mature miRNA accumulation in human tumors, and *XPO5* knockdown enhances tumor progression (Kumar *et al.*, 2007). Impaired miRNA biogenesis also escalates tumorigenesis, as DGCR8, Drosha, and Dicer knockout tumor cells proliferate rapidly and increase tumor growth over time (Zhang *et al.*, 2011b).

As miRNA profile changes are observed in many types of cancers, the use of miRNA detection as a biomarker for cancer, or anti-miRNA therapy deserves to be investigated. In addition to cancer, autoimmune diseases such as systemic lupus erythematosus (SLE) have shown that miRNA profiles differ in patient subsets with

different autoantibody specificities (Chauhan *et al.*, 2014). This is significant as SLE patients have heterogeneous clinical manifestations and identifying common subsets allows targeted treatment of these patients. In particular, Chauhan et al. found that patients expressing anti-dsDNA also had dysregulated miRNAs targeting cytokine signaling. This suggested that presence of anti-dsDNA could affect the immune system via the specific subset of dysregulated miRNAs. Cell-free circulating miRNAs also vary in profiles in response to disease states, while some studies suggest it as a blood cell-based phenomenon (Pritchard *et al.*, 2012). Use of anti-miRNA therapy such as locked nucleic acid (LNA)-modified oligonucleotides to knockdown levels of oncomirs could also decrease tumorigenesis (Orom *et al.*, 2006). The highly regulatory nature of miRNAs on cellular processes makes it a prime target for investigation, as miRNA profile expression marks a specific state of cancer tissue.

1.8 Eμ-*Myc* mouse model

Contribution of DDR with miRNA regulation to affect tumor progression in a hematological cancer places the Eμ-*Myc* transgenic mouse as an ideal mouse model to study in the context of how endogenous nucleic acids affect both mechanisms, resulting in tumorigenesis.

Mouse models have been used to study tumor development and in vivo immune responses (Anderson and Bluestone, 2005; Cheon and Orsulic, 2011; Croxford *et al.*, 2013; Lam *et al.*, 2014a). In particular, the Eμ-*Myc* mouse model is selected as the mouse overexpresses the *Myc* oncogene, in which *Myc* is associated with DNA damage (Adachi *et al.*, 2001; Vafa *et al.*, 2002). *Myc* expression induces ROS activity along with partial disruption of the DDR (Vafa *et al.*, 2002). *Myc*-induced replication stress via accelerated cell cycle passage also results in genomic instability, karyotypic abnormalities, and gene amplification (Felsher and Bishop, 1999). Further, *Atm*-deficient Eμ-*Myc* mice display defective DDR and accelerated

lymphoma growth. This results in lower survival rates as compared to Eμ-*Myc* mice, suggesting a DDR-dependent tumorigenesis of these mice (Reimann *et al.*, 2007). This finding was extended to solid malignancies as inactivated *Atm* also accelerated tumorigenesis in a K5 *Myc* mouse model exhibiting *Myc*-overexpressed squamous epithelium (Pusapati *et al.*, 2006).

In addition to the effects of DNA damage on Eμ-*Myc* mouse tumorigenesis, oncogenic non-coding miRNAs also modulate tumor formation. An example of oncogenic miRNAs is the *Mir-17-92* cluster, of which its overexpression correlates with reduced apoptosis and aggressive tumors in Eμ-*Myc* mice (He *et al.*, 2005). The c-*Myc* oncogene has been shown by chromatin immunoprecipitation to bind directly to the *mir-17* cluster locus, providing strong indication of direct regulation of miRNAs by c-*Myc* (O'Donnell *et al.*, 2005).

Eμ-*Myc* mice develop B cell lymphomas by day 60 of age, and they display phenotypic similarity to human acute lymphoblastic leukemia (ALL) (Adams *et al.*, 1985; Harris *et al.*, 1988). The c-*Myc* oncogene of Eμ-*Myc* mice is under the control of the immunoglobulin heavy chain enhancer (Eμ) and this dysregulation is genetically homologous to that of human Burkitt lymphoma (Adams *et al.*, 1985). Since *Myc* dysregulation is found in 70% of human malignancies, as reviewed by Meyer and Penn (Meyer and Penn, 2008), the Eμ-*Myc* mouse model is therefore important in the study of tumorigenic malignancies.

One of the major characteristics of the Eμ-*Myc* transgene-hemizygous mice is the early accumulation of polyclonal tumorigenic B lymphocytes, following which there is sharp regression in the periphery but not bone marrow of mice between 6-8 weeks of age (Harris *et al.*, 1988; Sidman *et al.*, 1993). Eventually B-cell lymphoma develops at the late stage by 15-20 weeks of age, where the prior regression is attributed to early anticancer immune signaling. A small subset of aged mice over 14 weeks of age had lower pre-B cell levels in spleen and bone marrow as compared to younger Eμ-*Myc* mice (Langdon *et al.*, 1986). Our group has examined the

regression phase of tumor cells and shown increased NK and T-cell immunosurveillance, along with activation of the DNA damage response and DNAM-1 ligand expression, resulting in spontaneous rejection of these tumor cells in Eμ-*Myc* mice (Croxford *et al.*, 2013).

1.9 Aims

While cytosolic nucleic acids have been largely characterized with respect to viral and bacterial infections, less is known about the presence of cytosolic nucleic acids in non-infected human cancer cells and their potential role in cancer progression. The following hypotheses drive the formation of three broad areas in my research project:

a. dsDNA in the cytosol of human tumor cells is dependent on DDR, similar to that in mouse tumor cells.
b. Cytosolic RNA:DNA hybrids exist in human tumor cells and are regulated by mechanisms that may be similar to cytosolic dsDNA.
c. The tumor regression phase of the Eμ-*Myc* mouse model is activated by TBK1 signaling pathway of innate immune sensing.
d. This sensing results in the production of proinflammatory cytokines such as TNFα to initiate an anti-tumor response.

Thus, the three broad areas (illustrated in Fig. 1.7) that my research entails are:

1. Characterization of dsDNA in human tumor cells;
2. Discovery of RNA:DNA hybrids in human tumor cells and mechanisms of generation;
3. Phenotypic characterization of *Tbk1*-deficient Eμ-*Myc* mice and *Tnfα*-deficient Eμ-*Myc* mice (Generation of these mice was carried out in Japan.)

Therefore, the aim of my PhD project is to characterize the presence of dsDNA and RNA:DNA in the cytosol of human cells, and to investigate the molecules involved in their interaction, thus revealing a possible role of these nucleic acid structures. As part of the research on the role of cytosolic DNA in tumor sensing and progression, it is appropriate to study the role of TBK1 and TNF-α using the Eμ-*Myc* tumor mouse model. This study contributes to the field of DNA-sensing and the immune response in tumorigenesis by extending the scope of nucleic acids that may play a role in the above-mentioned processes.

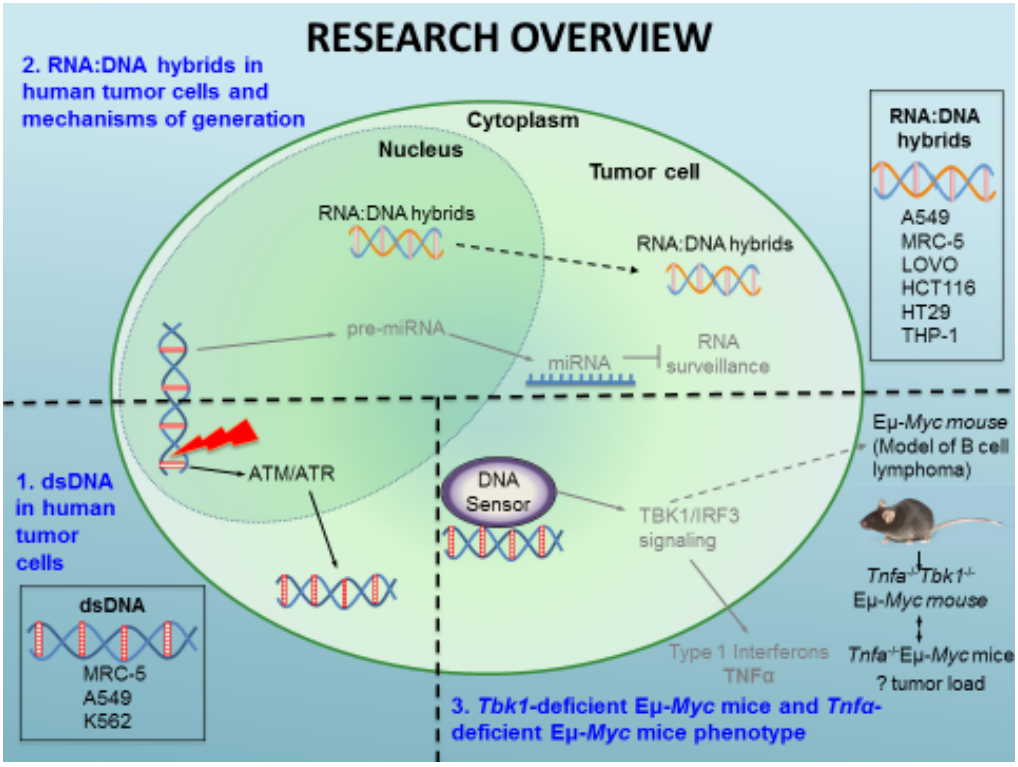


Figure 1.7 Overview of the thesis areas of research. The three broad areas of research are numerically listed (blue) in the schematic diagram: 1) The study of cytosolic dsDNA in human tumor cells, 2) the discovery of RNA:DNA hybrids and its characterization, and 3) Phenotypic characterization of *Tbk1*-deficient Eμ-*Myc* mice and *Tnfα*-deficient Eμ-*Myc* mice .

Chapter 2: Materials and Methods

2.1 Mice and cells

C57BL/6 mice were purchased from the Centre for Animal Resources at Osaka University. Eμ-*Myc* transgenic mice on a C57BL/6 background were purchased from Jackson Laboratory (USA). *Tnf*$^{-/-}$ mice on a C57BL/6 background and *Tnf*$^{-/-}$*Tbk1*$^{-/-}$ mice on a 129/Ola3C57/BL6 background were provided by Prof. K. Ishii (National Institute of Biomedical Innovation, NIBIO) and Prof. S. Akira (iFREC, Osaka University), as described previously (Hemmi *et al.*, 2004; Ishii *et al.*, 2006; Ishii *et al.*, 2008). Mice were bred and housed according to the guidelines by the National Institute of Biomedical Innovation. The human colorectal adenocarcinoma (LOVO and HT29), colorectal carcinoma (HCT116), lung adenocarcinoma (A549), monocytic cell lines (THP-1) were purchased from ATCC (USA). Cells were grown in Dulbecco's modified Eagle's medium (Nacalai Tesque, Japan), supplemented with 10% fetal bovine serum (Cell Culture Bioscience, Japan), 1% penicillin/streptomycin (Nacalai Tesque), and 2% HEPES (Life Technologies, USA). Cells were maintained with 5 μg/ml of Plasmocin (Invivogen, USA) to prevent mycoplasma infection.

2.2 Reagents

Cytarabine (Ara-C) was purchased from (Wako Chemicals, Japan). RNA POL III inhibitor, ML-60218 was purchased from Calbiochem (Japan). Both Ara-C and ML-60218 were dissolved in DMSO. ATM inhibitor, KU60019 (Tocris Bioscience, United Kingdom) and ATR inhibitor, VE821 (Axon Med Chem, Netherlands) were used at 10 μM. PicoGreen dsDNA reagent (Life Technologies, USA) was used at a 1:100 dilution. Mitotracker (Life Technologies) was dissolved in DMSO and used at 500 nM. Fixed cells were treated with 0.5 U/ml recombinant RNase H (NEB, USA) for 3 hrs at 37°C.

2.3 Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 10 min, and permeabilized in 0.2% Triton X-100 for 15 min. Non-specific sites were blocked with 2% goat serum and 1% BSA in 0.2% Triton X-100 for 1 h. Transfected cells were stained with anti-COX IV antibody (ab16056, Abcam, United Kingdom), anti-POLR3G (LS-C163858, LS Bio, USA), or anti-DDX17 (19910-1-AP, Proteintech, USA). The RNA:DNA hybrid-specific S9.6 antibody was a kind gift of Dr. D. Koshland, University of California, Berkeley (Boguslawski *et al.*, 1986). Secondary polyclonal antibodies used were Alexa Fluor AF488 F(ab')2 Fragment of Goat Anti-Mouse IgG (H+L) (Life Technologies) and Alexa Fluor AF555 F(ab')2 Fragment of Goat Anti-Rabbit IgG (H+L) (Life Technologies). PicoGreen staining of DNA and MitoTracker Red CM-H2XRos staining of mitochondria were performed according to the manufacturer's instructions (Life Technologies). Cells were stained with 2 μg/ml of Hoechst for 10 min and mounted in mounting medium (Dako, USA). Cell images were taken with a Leica TCS SP2 laser confocal scanning microscope (LCSM), and analyzed using Volocity (Version 6.2.1) and Imaris. Micrographs showed cells representative of total cell populations.

2.4 Electron microscopy

Cells were fixed in 4% paraformaldehyde buffered in 0.1 PB at 4°C overnight, dehydrated with ethanol before sectioned into 100nm-thin sections. Sections were first treated with RNase A for 1 hr, at 37°C, stained with 10 μg/ml of dsDNA antibody, before binding to mouse IgG conjugated with 50 nm colloidal gold particles (GE Healthcare). For immunogold labeling, cells were fixed in 2% glutaraldehyde in PBS at 4°C overnight, and in subsequently in 1% osmium tetroxide. Images were made on a transmission electron microscope (H-7650, Hitachi, Tokyo, Japan).

2.5 ELISA

96-well polystyrene plates were blocked with 1% BSA for 1 hr and subsequently coated with 2, 5, or 10 μg/ml of calf thymus DNA, Poly A:U, Poly G:C or Poly I:C in 25 μl PBS buffer for 18 hrs at 4°C. Some wells were treated with 1 mg/ml DNase I (Roche) for 1 hr. After washing with 0.1% Tween-20 PBS, anti-dsDNA antibody (Millipore), or anti-dsDNA antibody pre- incubated for 30 min with calf thymus DNA was added in triplicates and incubated for 1 hr at 37°C followed by goat anti-mouse IgG3-HRP (Southern Biotech) for 1 hr at 37°C. The reaction was visualized by TMB Microwell Peroxidase Substrate System (KPL). IFNβ concentration in the supernatant of treated cells were measured according to the manufacturer's instructions (PBL InterferonSource).

2.6 Immunoblot

Cells were lysed in cold RIPA buffer (Nacalai Tesque) and lysates were electrophoresed in 4-12% NuPAGE Bis-Tris gel (Life Technologies) before blotted onto PVDF membranes. Antibodies specific to DDX17 (sc-86409, Santa Cruz, USA), AGO2 (C34C6, Cell Signaling Technology, USA), and GAPDH (M171-3, MBL), and horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) were used to develop the blots, with Immobilon Western Chemiluminescent HRP Substrate (Milipore, Germany). Digital images were acquired using ImageQuant LAS 500 (GE Healthcare, United Kingdom).

2.7 Immunoprecipitation and mass spectrometry

2 x 10^6 A549 cells were seeded into 100-mm dishes and fixed in 1% paraformaldehyde (Nacalai Tesque) for 10 min, followed by treatment with 125 mM of Glycine (Wako, Japan) for 5 min. Cells were fractionated using MitoSciences cell fractionation kit (MS861, Mitosciences, USA). The cytosolic fraction was precleared by incubation with 5 μl of Protein G–Sepharose beads (GE Healthcare) at 4°C for 20 min on a rolling shaker. The cleared supernatant was incubated at 4°C overnight on a

rolling shaker with 10 μg/ml of RNA:DNA hybrid antibody and 10 μl of Protein G–Sepharose beads. Immunoprecipitates were washed subsequently with RIPA buffer, low salt buffer (20 mM Tris-HCL pH 8.1, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2mM EDTA), high salt buffer (20 mM Tris-HCL pH 8.1, 600 mM NaCl, 0.1% SDS, 1% Triton X-100, 2mM EDTA), final wash (20 mM Tris-HCL pH 8.0, 0.1% SDS, 1% Triton X-100, 1 mM EDTA) and TE buffer. Beads were resuspended in TE buffer with 1% SDS and incubated at 65°C overnight to release protein complexes for subsequent gel electrophoresis. For mass spectrometry, similarly processed cell lysates were immunoprecipitated with RNA:DNA hybrid antibody, and silver stained using Silver Stain Plus Kit (Bio-Rad, USA) according to the manufacturer's instruction. Bands of interest were cut out and sent for mass spectrometry analysis at the Osaka University mass spectrometry facility.

2.8 Transfection

A549 cells were transfected with siRNA of *DDX17* or *POLR3G* (Qiagen, Netherlands) using LipoFectamine 2000 (Invitrogen) according to manufacturer's instructions. AllStars Negative Control siRNA (Qiagen) was used as a control transfection and the sequence was proprietary. The siDDX17 sequences used were: siDDX17#1, 5'-CTGGAGTGCATTTGATAGTTA-3', siDDX17#2, 5'-CGGGATCG TAGTGAAACCGAT-3', and siDDX17#3, 5'-CAGATCTGATATGGGACTATT-3'. The siPOLR3G sequences used were: siPOLR3G#1, 5'-AAGGCACACCACTCACT AATA-3' and siPOLR3G#2, 5'-TTGCGGTTGATAATTAACATA-3'.

2.9 MiRNA microarray analysis

A549 cells were treated with 10 μM of RNA POL III inhibitor for 24 hrs, or subsequently treated with 10 μM of cytarabine or DMSO for 15 hrs. DMSO-treated cells served as a control. Total RNA was extracted by Trizol (Life Technologies) and labeled using a 3D-Gene miRNA labeling kit. The labeled RNA was hybridized to a human miRNA V19 microarray chip containing 2019 miRNA probes and analyzed

on a ProScanArray™ microarray scanner (Toray Industries, Japan). MiRNA profiles from two pairs of experiments (DMSO, and RNA POL III inhibitor-treatment conditions with and without Ara-C each) were provided as sample-wise median-normalized data by Toray. Data from these four profiles were further normalized using all-sample quantile normalization protocol using the corresponding Bioconductor package developed by Bolstad et al. (Bolstad *et al.*, 2003). Original miRNA profiles consisted of 2019 miRNA probes, of which only a small fraction showed significant expression in any of these experiments. After replacing the missing valued data (no expression observed) by the minimum of all observed expression values, miRNA probes that showed at least 3-fold differential expression between any pair of 4 experiments, were used for further quantitative analysis. Identified miRNA sequences were used to obtain predicted gene targets, as acquired from public domain resource, mir-DIANA (Vlachos *et al.*, 2012). A P-value threshold of 0.05 and MicroT threshold of 0.8 was applied.

2.10 Quantitative PCR

Total RNA was isolated using the RNeasy kit (Qiagen, Netherlands). Real-time PCR assays were performed using an Applied Biosystems 7500 sequence detector. 2 µg of total RNA was reverse transcribed to cDNA using random hexamers and a SuperScript II First-Strand Synthesis System (Invitrogen, USA). Each amplification mixture (25 µl) contained 25 ng of reverse-transcribed RNA, 8 mM forward primer, 8 mM reverse primer and 12.5 µl of iTaq SYBR Green Supermix with ROX (Bio-Rad). PCR thermocycling parameters were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15s, 60°C for 15s and 72°C for 1 min. All samples were normalized to the signal generated from the housekeeping gene *HPRT1*. SYBR green PCR was performed in triplicate. The following primers were used: *XPO1*-5', 5'-AGGTTGGAGAAGTGATGCCA-3'; *XPO1*-3', 5'-GCACCAATCATGTACCCC AC-3'; *KPNB1*-5', 5'-GACCGACTACCCAGACAGAG-3'; *KPNB1*-3', 5'-GACTC

CTCCTAAGACGACGG-3'; *NUP153*-5', 5'-GCCCAAATCTTCCTCTGCAG-3'; *NUP153*-3', 5'-GAAAGGAGCCACTGAAGCAC-3'; *HPRT1*-5', 5'-CCCTGGCGT CGTGATTAGTG-3'; *HPRT1*-3', 5'-TCGAGCAAGACGTTCAGTCC-3'. Samples prepared without reverse transcription served as negative control templates.

2.11 Flow cytometry

Blood from mice was collected by facial bleeding and red blood cells were removed by red blood cell lysis. Fc receptors on blood cells were blocked by incubating cells with CD16/CD32-specific antibodies for 15 min (eBioscience, USA). Tumor cells were stained with B220-APC (clone RA3-6B2; eBioscience) and Immunoglobulin M (IgM)-FITC (clone RMM-1, BioLegend, USA). Staining of cells was analyzed using a BS LSRII (BD Biosciences, USA) and FlowJo. 8.8.7 (TreeStar, USA). Tumor load was calculated as follows: Σ (% IgM−B220low) × % B220++ (IgM+B220low) × % B220+.

2.12 Statistical Analysis

For statistical analysis, one-tailed Student's t-test ($P<0.05$) was used unless otherwise stated, after data were tested positive for normality by Shapiro-Wilk test. For data that failed normality test, non-parametric Mann-Whitney Wilcoxon Rank-Sum test was used. Error bars represent standard error unless otherwise stated in the figure legends. Mice survival data were analyzed using the Log-rank test and by Gehan-Breslow-Wilcoxon test (specific statistical analysis for comparison of survival data). Results that were statistically significant with a P-value of < 0.05 were represented by * in the figures.

Chapter 3: Presence of DNA in the Cytosol in response to DNA damage

3.1 Presence of cytosolic DNA in cell lines

Genomic DNA normally locates in the cell within the nucleus or mitochondria, while some retroelements locate in the cytosol (Rush and Misra, 1985). However, genomic stability in cancer cells with DNA breaks may cause the presence of delocalized nucleic acids. Previously, Lam et al., used dsDNA antibodies to stain for the presence of dsDNA in Eμ-*Myc* derived murine tumor cells (Lam *et al.*, 2014a). It was hypothesized that presence of dsDNA was associated with tumorigenesis of cancer cells, since dsDNA had been previously shown to trigger an antiviral immune response (Ishii *et al.*, 2006).

We first confirmed the specificity of the cytosolic DNA antibody with a series of DNA ELISA titrations. dsDNA antibody had a high affinity to calf thymus (CT) dsDNA, and competitive inhibition from increasing concentrations of free CT-DNA decreased binding affinity of dsDNA antibody (Fig. 3.1a). DNase I treatment of coated DNA before incubation with dsDNA antibodies abrogated the binding affinity of the dsDNA antibody, suggesting the presence of DNA being removed by DNase I treatment and hence antibody binding. CT DNA showed the highest affinity for dsDNA antibody as compared to other synthetic polynucleotides, and Poly I:C, a synthetic analog of dsRNA (Fig. 3.1b). IgG3 anti-mouse antibody was used as a control to the dsDNA antibody, and showed no observable binding to CT DNA (Fig. 3.1c).

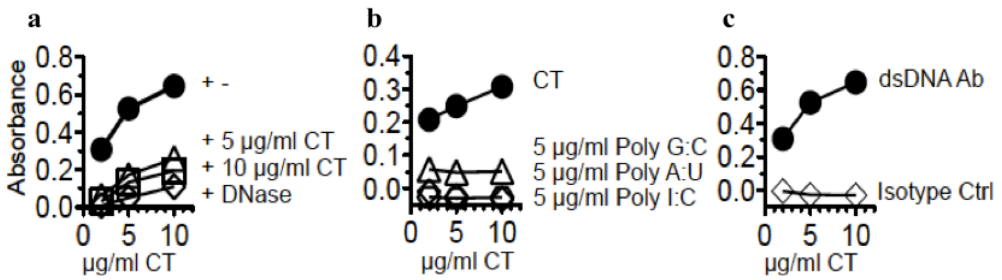


Figure 3.1 Specificity of dsDNA antibodies. Anti-dsDNA mouse monoclonal antibody preferentially recognizes ds-nucleotides. ELISA plates were coated with indicated amounts of calf thymus DNA 94. Coated plates were incubated with dsDNA-specific antibody (a, b, c) or isotype control antibody (c). As a control, some wells were incubated in the presence of (a) calf thymus DNA (CT), DNase I, or (b) Poly G:C, Poly A:U, Poly I:C. Specific antibody staining was visualized by ELISA.

To determine if cytosolic dsDNA was also present in tumor cells, various human cell lines were stained with anti-dsDNA antibodies. Fluorescent microscopy confirmed the presence of cytosolic dsDNA (Fig. 3.2), congruent with observance of cytosolic dsDNA present in murine tumor cells. To exclude double-stranded mitochondria DNA, cells were co-stained with COX IV, a mitochondrial membrane protein. Further, cloning and sequencing of the DNA revealed nuclear repetitive sequences (Shen et al. manuscript under revision) indicative of DNA fragments originating from nucleus.

To characterize the staining location of dsDNA, MRC-5 cells, which had a relatively large cytoplasmic area for visualization, were immunogold labeled using dsDNA antibodies, and viewed with electron microscopy. Fixed cells were pre-treated with RNase A to remove unspecific dsRNA staining. Comparing against the isotype antibody, significant staining of the cytoplasm and nucleus was observed with dsDNA antibodies, visualized as black circular particles (Fig. 3.3). However, the dsDNA did not concentrate at a particular organelle or surface membrane, suggesting the presence of dsDNA in the cytosol independent of compartmental organelles.

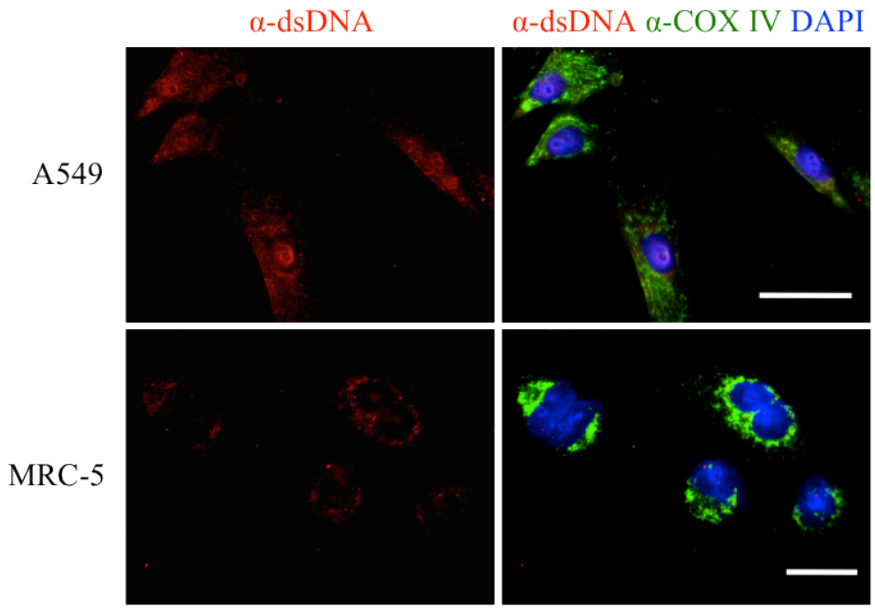


Figure 3.2 Presence of cytosolic DNA in various cell lines. The human lung carcinoma cell line A549 and human lung tissue derived cell line MRC-5 was stained with dsDNA-specific antibodies (Red), COX IV-specific antibodies (Green), and DAPI (Blue).

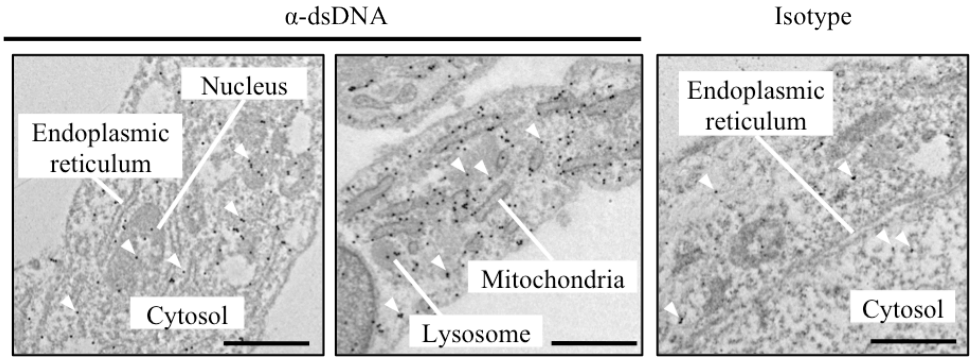


Figure 3.3 Staining of cytosolic dsDNA in MRC-5 cells. MRC-5 cells were pre-treated with RNase A, fixed and sectioned before staining by immunogold labeled dsDNA antibodies. These were visualized as black circular particles throughout the cell under transmission electron microscopy. Representative black particles were indicated by white arrows. Scale bars represent 10 μm.

3.2 DNA damage increases level of cytosolic DNA

To investigate the effect of DNA damage on cytosolic DNA, A549 cells were treated with cytarabine (Ara-C). Ara-C is a chemotherapy drug commonly used in leukemias such as acute myeloid leukemia (AML) and non-Hodgkin lymphoma was used to treat cells (Wiernik *et al.*, 1989). Ara-C damages DNA by interfering with DNA synthesis during the S phase of cell replication, exhibiting anti-metabolic activity by mainly masquerading as a pyramidine (cytosine arabinoside triphosphate) for incorporation into the extending DNA strand (Jamieson *et al.*, 1990; Gmeiner *et al.*, 1998). Previous treatment kinetics studies with Ara-C suggested IRF3 phosphorylation for interferon response occurred by 15 hrs of Ara-C treatment (Lam *et al.*, 2014a). To determine if similar kinetics might occur with cytosolic DNA accumulation after DNA damage, cytosolic dsDNA was quantified using a Qubit assay, normalizing dsDNA concentration to overall protein levels. This assay uses a dsDNA dye that only fluoresces when bound to DNA, and fluorescence is read via a fluorometer. With a time-course treatment of Ara-C from 0-15 hrs, increasing concentration of dsDNA was detected in cytosolic fractions by Qubit assay, while dsDNA concentration in nuclear fractions did not increase significantly (Fig. 3.4a). At 24 hrs, cells could be going through apoptosis due to high toxicity of Ara-C over an extended period of time, thus the dsDNA might be fragmented and removed from the cell. To assess purity of samples, a western blot of each fraction was run and stained with cytosolic and nuclear protein markers, which indicated the existence of dsDNA in the pure cytosolic fraction (Fig. 3.4b).

To visualize this increase of cytosolic dsDNA, cells were first treated with Ara-C before dsDNA antibody or PicoGreen staining. DMSO treatment was used as a negative control, since DMSO was used as a solvent for Ara-C. PicoGreen is a DNA staining dye that intercalates double strand DNA and also interacts with the minor groove of DNA (Dragan *et al.*, 2010). In addition, p-ATM was co-stained to detect

for the occurrence of the DDR in response to damage, which activates downstream effector molecules through phosphorylation. ATM (ataxia talengiectasia mutated) is phosphorylated at Ser 1981 in response to DNA damage, which stabilizes ATM to provide its activation of kinase activity to initiate a proper DNA damage response (So *et al.*, 2009). Increased p-ATM nuclear staining of cells after treatment with Ara-C indicated cellular DNA damage (Fig. 3.5a).

Similar to the Qubit assay, presence of dsDNA in the cytosol increased with DNA damage, as shown by increased cytosolic staining by dsDNA antibody (Fig. 3.5, a and c), and PicoGreen (Fig. 3.5, b and d) in cells after Ara-C treatment. In addition, other cell lines such as LOVO and HCT116 colorectal adenocarcinoma also show similar cytosolic DNA staining (Shen et al., manuscript in preparation). These data collectively indicate the importance of DNA damage for cytosolic dsDNA accumulation in human tumor cells.

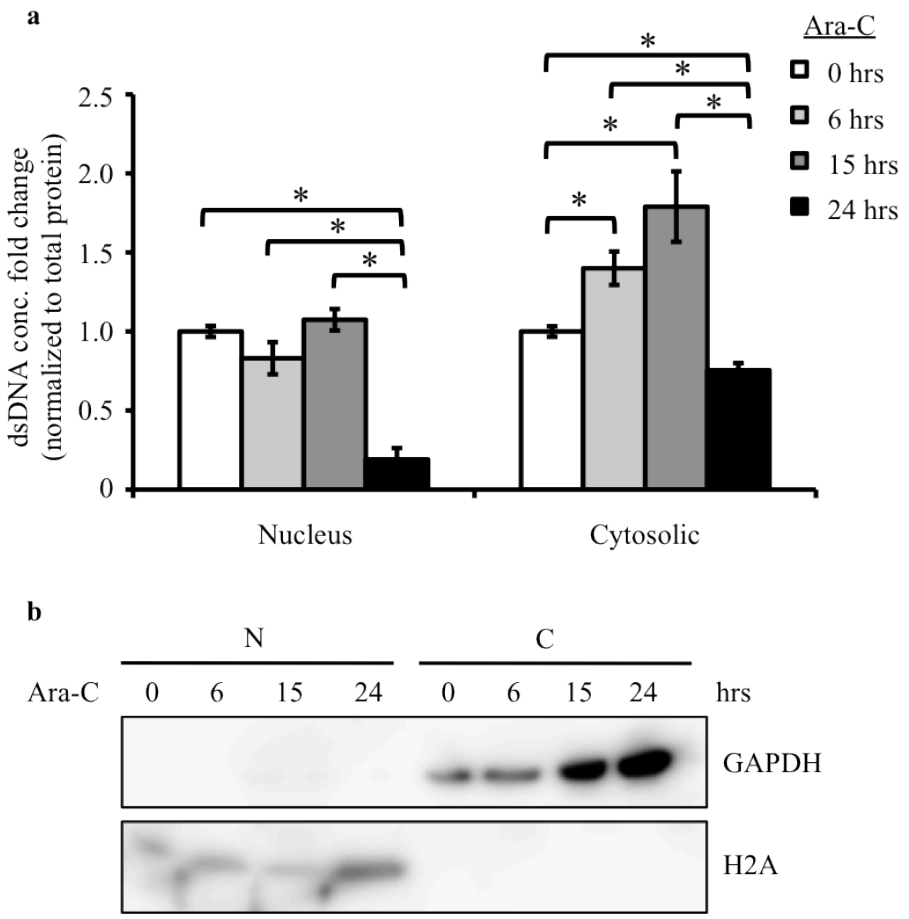


Figure 3.4 Cytosolic DNA increased in response to DNA damage. (a) A549 cells were treated with DMSO or 10 μM Ara-C for increasing periods of time (0, 6, 15, 24 hrs). Cells were then fractionated into nuclear and cytosolic fractions. dsDNA and protein concentration was measured using the Qubit assay. Data were plotted with (mean ± SEM), * $P < 0.05$. (b) A portion of the obtained fractions from (a) were used for immunoblotting, and staining with antibodies against GAPDH and histone H2A.

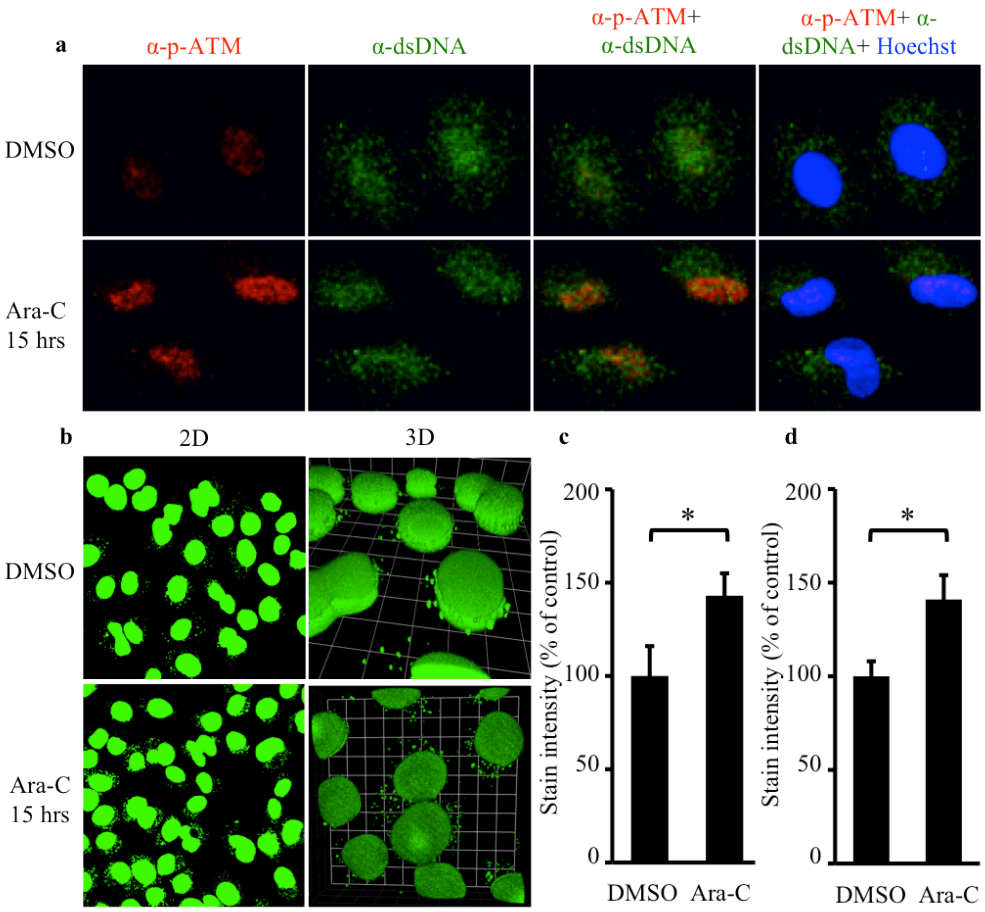


Figure 3.5 Presence of cytosolic DNA increases after DNA damage with Ara-C. A549 cells were either treated with DMSO or 10 μM of Ara-C for 15 hrs. (a) Cells were stained for marker of DDR p-ATM (Red), dsDNA antibodies (Green), and Hoechst (Blue). (b) Cells were stained with 10 μl/ml of the vital dsDNA-specific dye PicoGreen (Green) for 1 hr. (c and d) Quantification of cytosolic dsDNA antibody stain intensity in (a) and cytosolic PicoGreen stain intensity in (b) respectively. Data were plotted with (mean ± SEM), * $P < 0.05$.

3.3 Accumulation of dsDNA by DNA damage depends on ATM

DNA damage response involves p53 activation with the phosphorylation of ATM or ATR that activates Chk1. To examine the regulation of dsDNA by DNA damage, A549 cells were treated with an ATM/ATR inhibitor, caffeine, for increasing periods of time, and cytosolic dsDNA accumulation disappeared after up to 24 hrs of treatment (Fig. 3.6, a and c). This ATM/ATR-dependent inhibition was also seen in another cell line THP-1, derived from an acute monocytic leukemia patient (Fig. 3.6b). The same dsDNA antibody was used for staining of Fig. 4.6a and b, and only the secondary antibody conjugated to different fluorophores (AF488 for green and AF549 for red), thus the stain colours would not account for the variation in staining intensities in different cells over a period of time. Taken together, this demonstrated that the accumulation of dsDNA was dependent upon DNA damage response mechanisms, and further attributes the occurrence of cytosolic dsDNA to the presence of DNA damage within the genome.

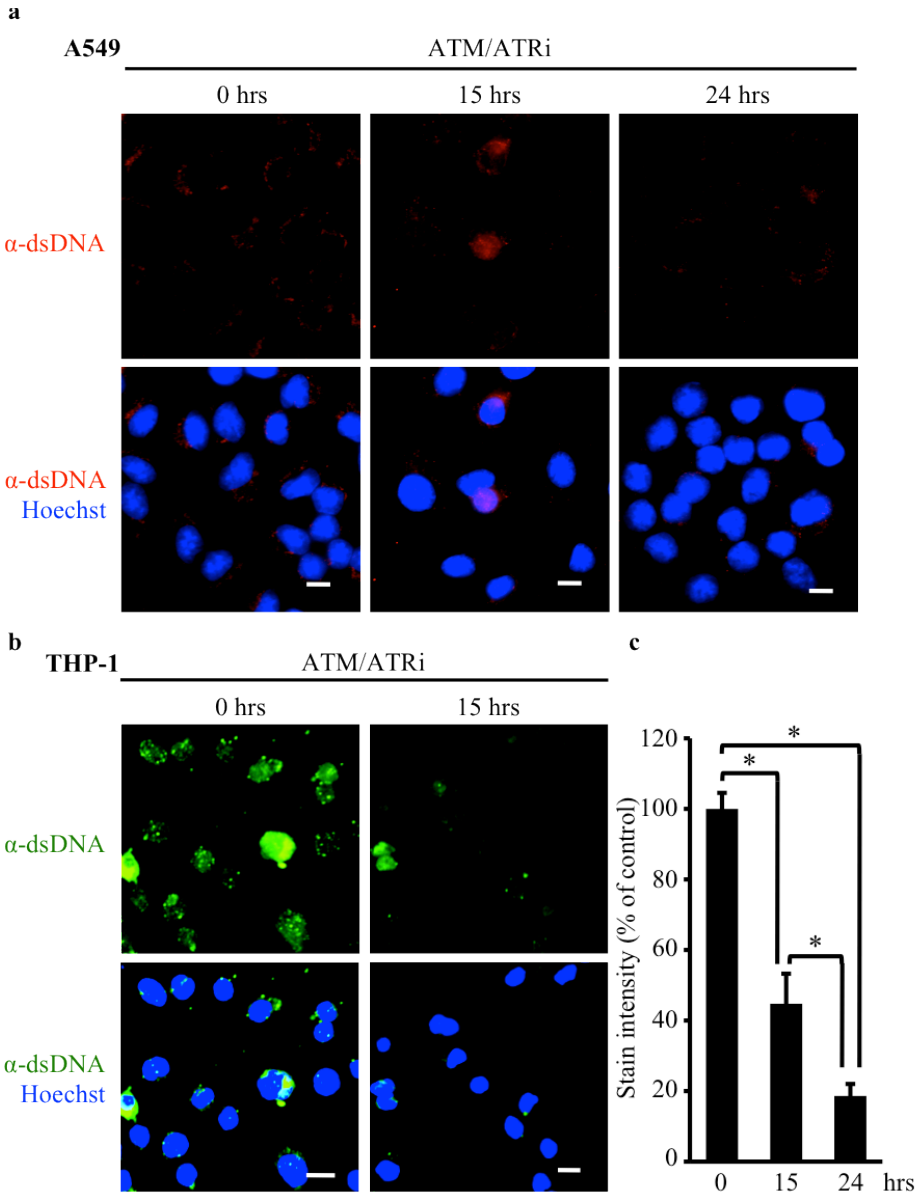


Figure 3.6 Cytosolic dsDNA disappeared after treatment with ATM/ATR inhibitor. (a) A549 cells were treated with 7.7 mM of ATM/ATR inhibitor, caffeine, for increasing periods of time (0, 16, 24 hrs), before being fixed and stained for dsDNA (Red), and Hoechst (Blue). (b) THP-1 cells were treated with 7.7 mM of ATM/ATR inhibitor, caffeine, for 15 hrs, before being fixed and stained for dsDNA (Green), and Hoechst (Blue). (c) Quantification of dsDNA stain in (a). Data were plotted as (mean ± SEM). * $P < 0.05$.

3.4 Cytosolic DNA does not colocalize with known DNA-binding molecules

To assess the significance of cytosolic DNA in DNA sensing, we examined the localization of cytosolic DNA-binding proteins with respect to cytosolic DNA. The antibodies were used in immunofluorescence staining with known cellular molecules to observe for colocalization.

HMGB1 is a DNA-binding protein involved in TLR9 signaling of CpG dsDNA in the cytosol (Ivanov *et al.*, 2007). In particular, HMGB1 is associated with DNA damage sensing, and was found to mediate the kinetics of p53 phosphorylation after Ara-C genotoxic agent exposure (Krynetskaia *et al.*, 2009). Knockout mice studies of HMGB1 have shown defects in type 1 interferon and cytokine production (Yanai *et al.*, 2009). We stained for HMGB1 along with dsDNA in MRC-5 cells, however HMGB1 was localized mainly in the nucleus, supporting previous reports of HMGB1 as a nuclear protein (Fig. 3.7).

DNA sensing also requires STING as a common modulator in many sensing pathways (Nakhaei *et al.*, 2010; Barber, 2014). STING has been suggested to localize to the mitochondria membrane, and moves to the endosome once activated. STING is able to bind cyclic-dinucleotides directly, and also functions as an indirect modulator of many dsDNA sensors, such as DDX41, IFI16, and cyclic GMP-AMP (cGAS) (Unterholzner *et al.*, 2010; Zhang *et al.*, 2011c; Lam *et al.*, 2014b). A possibility that cytosolic dsDNA could bind STING led us to co-stain dsDNA with STING. However, majority of the dsDNA did not colocalize with STING proteins in the cytosol (Fig. 3.7).

KU80 is a NHEJ complex protein that forms a heterodimeric complex with KU70, which has been purported to be a cytosolic DNA sensor that triggers type III interferon response to dsDNA (Zhang *et al.*, 2011a). Co-stain of KU80 with dsDNA in A549 cells showed that while KU80 had a mainly nuclear stain, there was a substantial cytosolic stain that did not colocalize with the cytosolic dsDNA antibody,

suggesting that the DNA repair DNA sensor KU70/80 in the cytosol was not bound to cytosolic dsDNA (Fig. 3.7).

Collectively, our data suggested that dsDNA was unable to be recognized by above-mentioned dsDNA sensors, raising the question whether cytosolic dsDNA in these human cell lines are able to invoke an innate immune response.

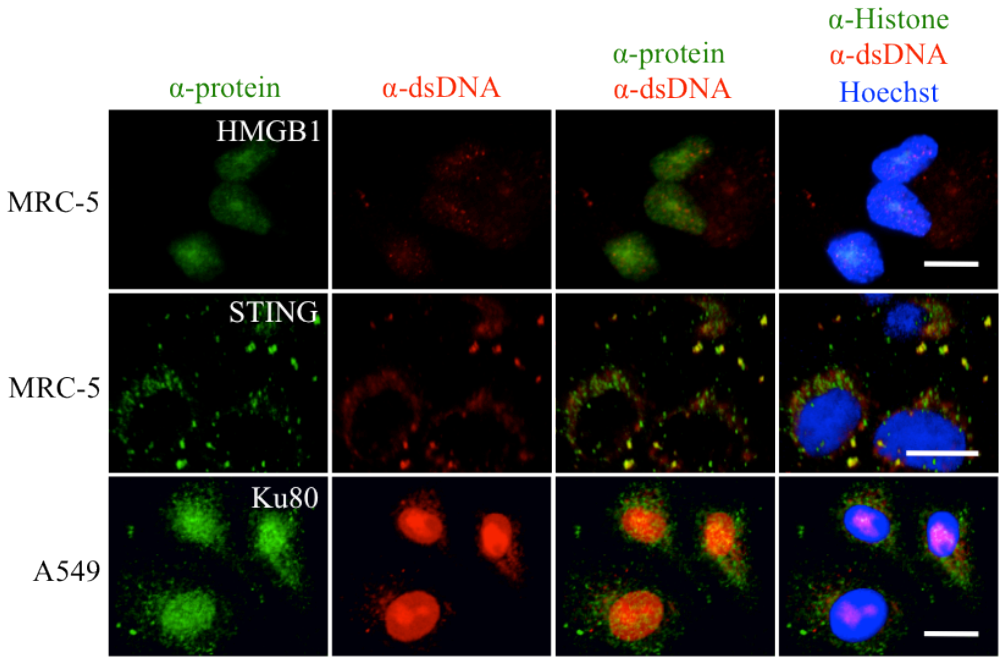


Figure 3.7 Cytosolic DNA partially colocalizes with DNA sensor molecules. A549 or MRC-5 cells were costained for dsDNA (Red), (a) HMGB1, (b) STING or (c) KU80 antibodies (Green) respectively, and Hoechst (Blue).

In addition to conventional DNA sensor proteins, histone H2B was identified to have anti-viral properties, as extrachromosomal histone H2B depletion resulted in decreased IFN-β production in HEK293 cells in response to dsDNA, and affected DNA virus replication (Kobiyama *et al.*, 2010). In particular, histone H2B is found both in the nucleus and cytoplasm, making it a possible candidate for the regulation of the cytosolic dsDNA. We also stained for additional histones in addition to H2B to detect for colocalization of dsDNA, namely H2A, H3, H3 (citrulline), and H4 (Fig. 3.8). In particular, nucleolar staining for some histones was also observed,

representative of chromatin regions in the eukaryotic cells in conjunction with chromosome unwinding from histones at sites of active transcription (Musinova *et al.*, 2011). H3 (citrulline) antibodies were used to stain citrullinated H3 proteins, as this posttranslational modification induced chromatin decondensation, which may interfere with transcription (Wang *et al.*, 2009; Sharma *et al.*, 2012). However, no distinct cytosolic stain was observed for any of the histone proteins.

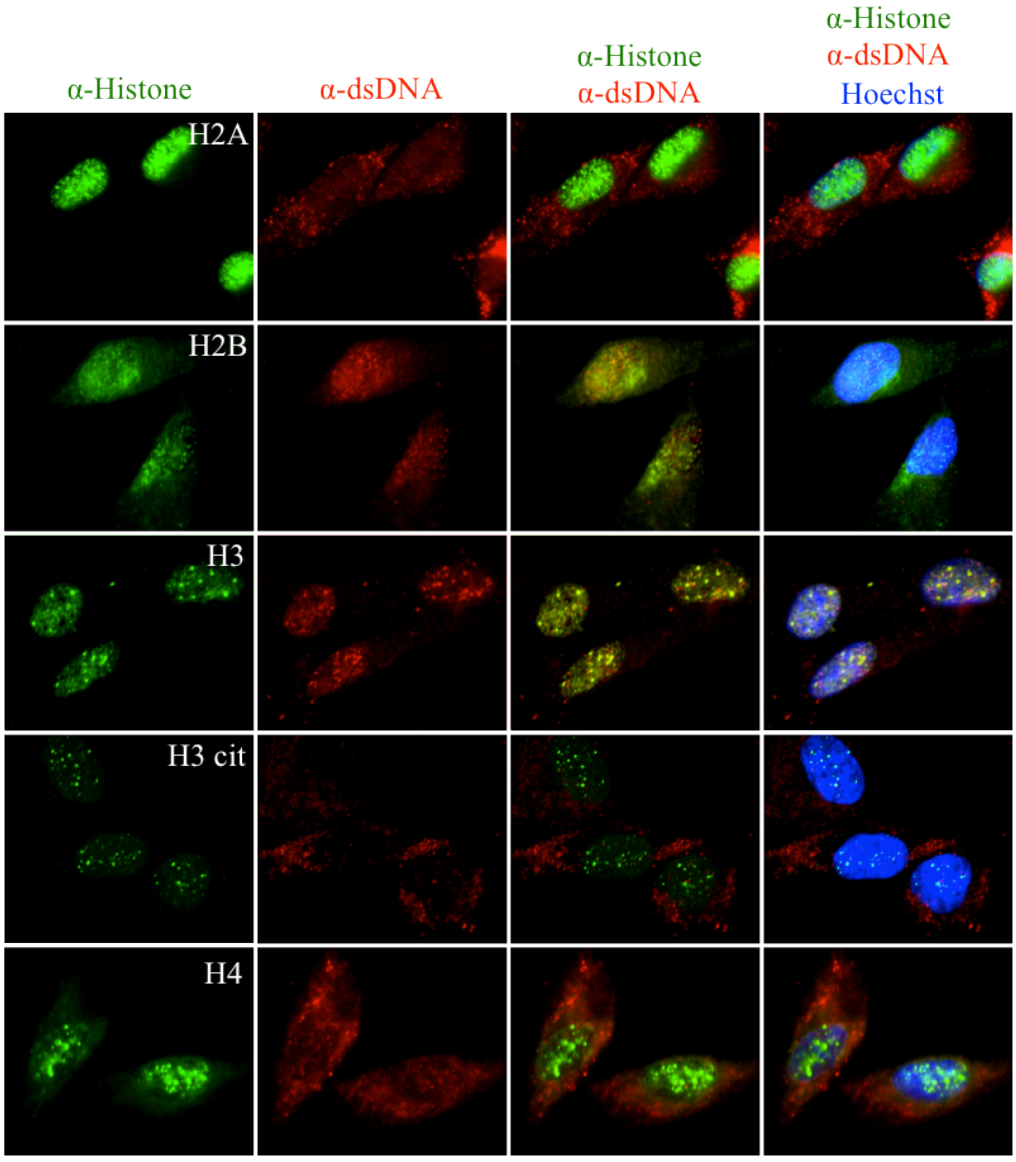


Figure 3.8 Cytosolic dsDNA does not colocalize with histone proteins. (a) MRC-5 cells were costained for histone proteins H2A, H2B, H3, H3 cit and H4 (Green), dsDNA antibodies (Red), and DNA using Hoechst (Blue).

To investigate if cytosolic DNA was located within organelles or complexes in the cell cytoplasm that might be less visible in prior electron microscopy experiments, we identified organelles that were associated with immune signaling or recognition of antigens. Exosomes are thought to promote immune responses (Anand, 2010; Bretz *et al.*, 2013). Recently, exosomal dsDNA (exoDNA) was found in tumor-derived exosomes, and exoDNA sequences were reflective of nuclear genomic regions, although lower amounts of exoDNA were observed in lung cancer cell lines (Thakur *et al.*, 2014). However, when we stained CD63, a known exosome marker, with dsDNA in MRC-5 lung-derived cells, we did not see significant costaining of the two (Fig. 3.9), suggesting a lack of interaction of dsDNA with exosomes.

Stress granules are cytoplasmic compartments that form in response to various types of stress (Tourriere *et al.*, 2003; Gao *et al.*, 2010). They contain RNA released from disrupted polysomes in response to stress, which stalls the translation process. Stress granule formation has also been thought to module the DNA damage response (Pothof *et al.*, 2009), providing a possibility that damage DNA might also associate with stress granules. Further, antiviral stress granules containing RIG-1 have been characterized (Onomoto *et al.*, 2012) after influenza A virus cellular infection. RIG-1 is a critical sensor of viral RNA that triggers the IPS-1 pathway to activate interferon-stimulated genes (Kawai *et al.*, 2005). We stained for G3BP1, a marker protein found in the stress granule complex, with dsDNA, however there was little colocalization of dsDNA cytosolic clusters and G3BP1 in MRC-5 cells (Fig. 3.9).

Autophagy is known to degrade damaged organelles and proteins, especially in cell starvation or damage (Mizushima, 2007). DNA damage also triggers autophagy, which might be an alternative route for a damaged cell, as opposed to apoptosis (Rodriguez-Rocha *et al.*, 2011). LC3B is a constituent of the autophagosome complex that forms as part of the autophagy process in the cytosol.

While we co-stained for LC3B and dsDNA, there was also no evidence for colocalization, suggesting that autophagy might not be involved in dsDNA accumulation in the cell (Fig. 3.9).

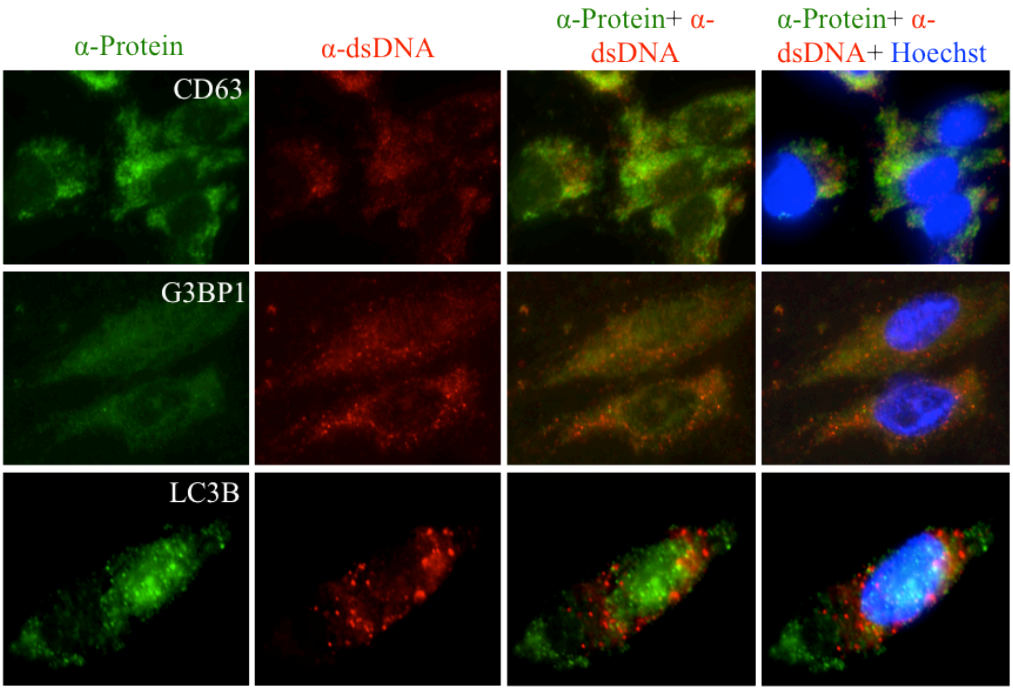
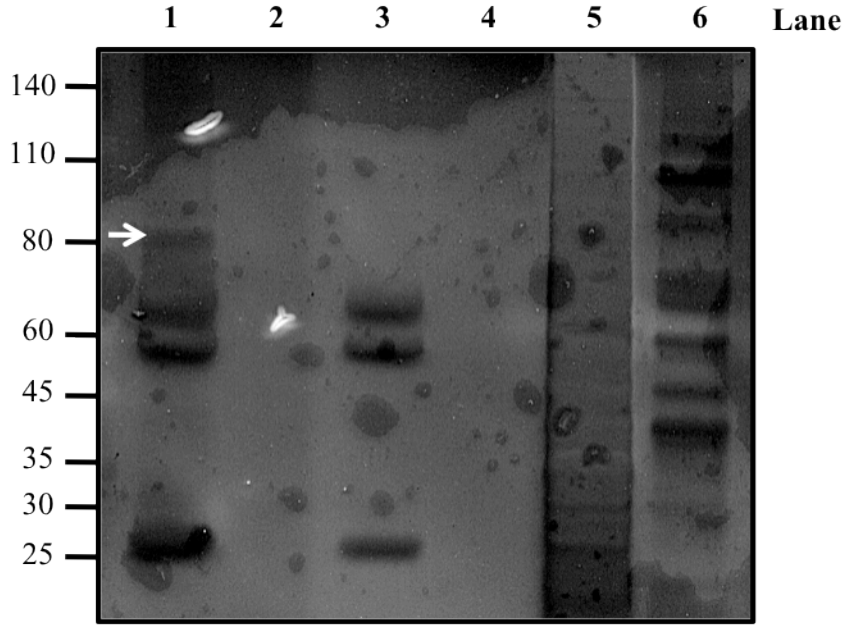


Figure 3.9 Cytosolic dsDNA does not colocalize with major compartmental proteins. MRC-5 cells were costained with antibodies specific against compartmental proteins CD63, G3BP1, and LC3B (Green) and dsDNA (Red) antibodies, followed by Hoechst (Blue).

3.5 DDX17 expression decreases with DNA damage

To investigate proteins involved in dsDNA binding, the cytosolic fraction of A549 cells was immunoprecipitated with dsDNA antibody and silver staining was performed on the SDS-PAGE gel. The immunoprecipitation was also performed with RNase H treatment, as RNase H is an enzyme that removes RNA:DNA hybrid specific interactions (Nowotny *et al.*, 2008; Rychlik *et al.*, 2010). A band of interest was identified by its disappearance after RNase H treatment (Fig. 3.10), as binding of these proteins to the dsDNA antibody would be sensitive to nucleic acid degradation. Mass spectrometry analysis of the cut band at 80 kDa revealed a list of potential proteins, of which one of the genes of interest was DEAD (Asp-Glu-Ala-Asp) Box

Helicase 17 (DDX17), a 72 kDa helicase protein. Further, DDX17 was identified to play a role in cell proliferation leading to cell survival. The possibility that cytosolic DNA could influence cell survival via the effect of cytosolic DNA led us to investigate the role of DDX17 in DNA damage.



Lane
1) dsDNA ab. IP (cytosolic)
2) dsDNA wash
3) dsDNA ab.+ Rnase H IP (cytosolic)
4) dsDNA+ Rnase H wash
5) Nuclear lysate
6) Cytosolic lysate

Figure 3.10 DDX17 potentially binds cytosolic dsDNA. A549 cells were fractionated and cytosolic fractions were immunoprecipitated with dsDNA antibody before RNase H or buffer treatment and subsequently SDS-PAGE analysis (Lane 1 and 3). The gel was silver-stained and the band of interest (white arrow) was sent for mass spectrometry analysis. Lane 2 and 4 were the final washes after elution of the protein to ensure that no protein remained on the beads. Lane 5 and 6 contained the fractionated A549 nuclear and cytosolic lysate respectively.

DNA damage is known to phosphorylate DDX17, possibly via ATM/ATR induction (Matsuoka *et al.*, 2007; Wang and Taniguchi, 2013). DDX5 was also shown to activate transcription factors such as Era, p53, and beta-catenin (Germann *et al.*, 2012). In addition to transcriptional regulation, DDX17 is known to be part of the

microRNA machinery in the nucleus, recognizing pre-miRNA seeded for export to the cytoplasm (Wang and Taniguchi, 2013). However, its specific role in miRNA regulation for tumorigenesis has yet to be elucidated (van Kouwenhove *et al.*, 2011). Nevertheless, DNA damage has been shown to regulate pre-miRNA export from the nucleus for miRNA processing in an ATM/Akt-dependent manner (Wan *et al.*, 2013).

To visualize the localization of DDX17, we stained A549 cells for DDX17 along with dsDNA. Majority of DDX17 accumulated in the nucleus (Fig. 3.11), consistent with the fact that DDX17 is a nuclear protein required in Drosha complex formation for miRNA biogenesis (Fukuda *et al.*, 2007). However, cytosolic DDX17 was present in cells, and upon treatment of DNA damage agent Ara-C, cytosolic DDX17 amounts were increased. This suggested that DDX17 could play a role in response to the DDR and the presence of cytosolic DNA.

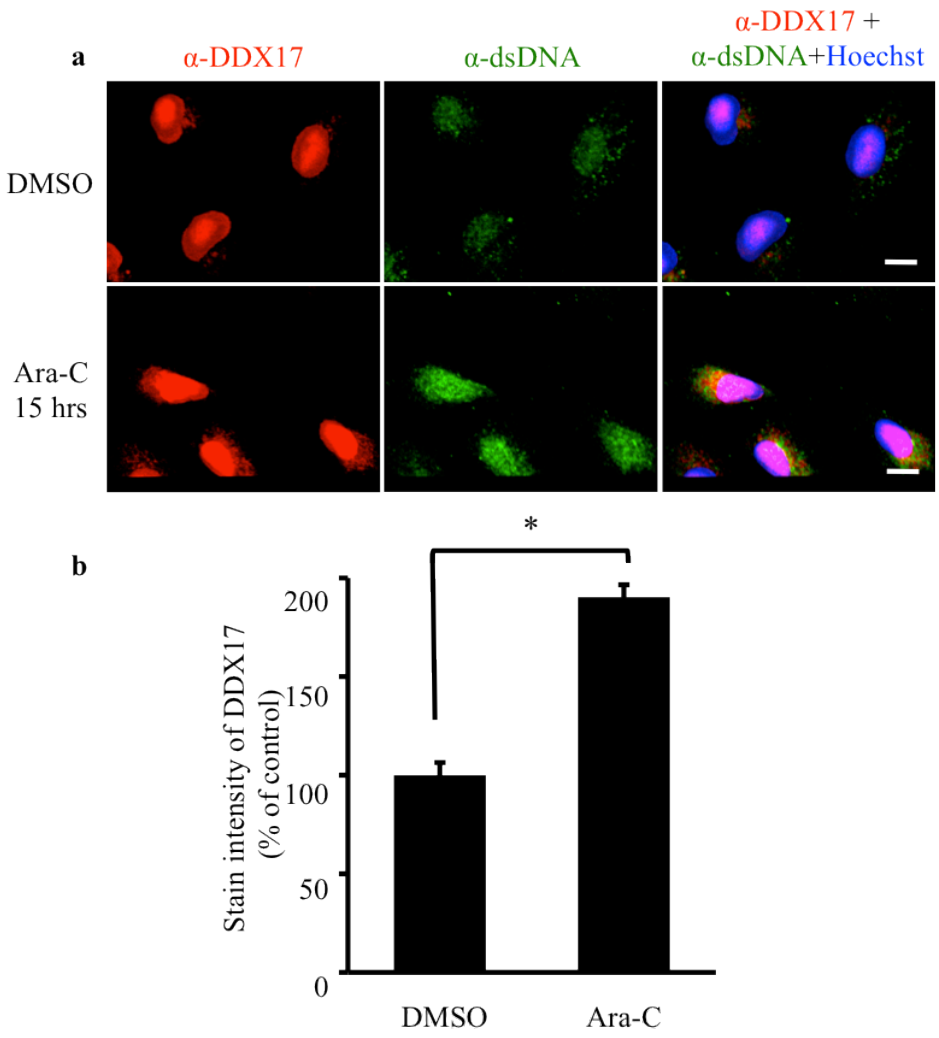


Figure 3.11 DDX17 is partially localized to the cytosol in response to DNA damage. (a) A549 cells were treated with DMSO or 10 μM of Ara-C for 15 hrs. Cells were stained with antibodies against DDX17 (Red), dsDNA (Green), and Hoechst (Blue). (b) Relative quantification of cytosolic DDX17 in (a). Two-tailed Wilcoxon test was performed. Error bars represent SEM. * $P < 0.05$.

3.6 DDX17 expression regulates the presence of cytosolic dsDNA

To assess if DDX17 may affect presence of cytosolic DNA, control or *DDX17* siRNA was transfected into cells, and fixed microscopy slides were treated with RNase A to remove unspecific double stranded RNA staining from siRNA. Negative control siRNA, a scrambled siRNA sequence determined not to target any gene, was used to controlled off-target effects of siRNA. Cells transfected with negative control siRNA were stained in the column labeled 'Control' and used for comparison of siDDX17. siRNA was transfected at a low concentration of 20 nM to reduce off-target effects that would be seen when cells are transfected at higher siRNA concentrations (>100 nM) (Semizarov *et al.*, 2003). Out of three siDDX17 sequences, siDDX17 #3 had the highest knockdown efficiency as observed by immunoblot (Fig. 3.12a). Immunofluorescence showed that knockdown of *DDX17* resulted in increased cytosolic DNA in A549 cells (Fig. 3.12b), suggesting that DDX17 activity was able to regulate the amount of cytosolic DNA.

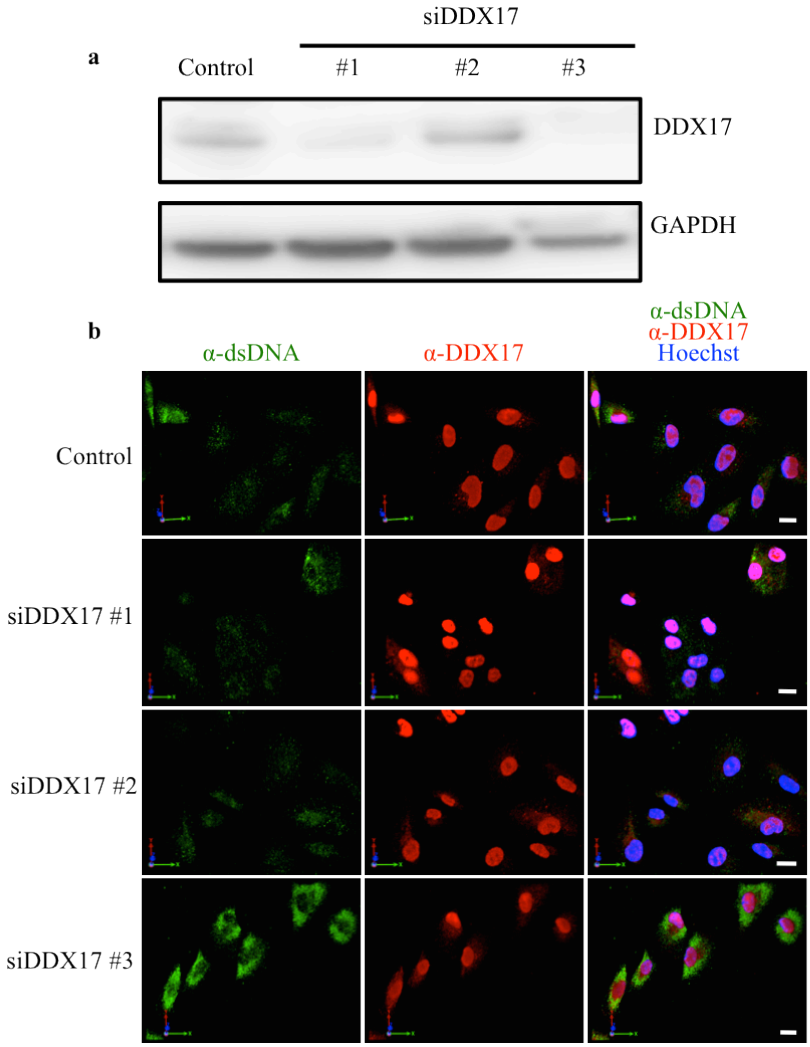


Figure 3.12 *DDX17* knockdown increased the levels of cytosolic DNA (a) A549 cells were transfected with 20 nM of control scrambled siRNA, or siDDX17 (#1, #2, #3). Cells were re-transfected with 20 nM of the same siRNA after 48 hrs to ensure sufficient knockdown. A portion of the transfected cells was used for immunoblot analysis of protein knockdown of DDX17. GAPDH was used as a loading control. (b) Cells were harvested two days after the second transfection for immunofluorescence staining using antibodies specific against DDX17 (Red), dsDNA (Green), and Hoechst (Blue).

Chapter 4: RNA:DNA hybrids in cytosolic abundance

4.1 Presence of RNA:DNA hybrids in cytosol

Lam et al previously stained for the presence of cytosolic DNA in murine tumor cells (Lam *et al.*, 2014a). Intriguingly, PicoGreen, the vital dye that was used in the detection of dsDNA (Ashley *et al.*, 2005; Dragan *et al.*, 2010), was also purported to detect RNA:DNA hybrids, as it also stains dsRNA with ten-fold lower intensity (Singer *et al.*, 1997). In an effort to characterize cytosolic DNA and the possibility of RNA:DNA hybrids in cells, the accumulation of cytosolic nucleic acids in various cells was investigated. PicoGreen staining in A549 human lung carcinoma cell line showed the presence of extranuclear nucleotides (Fig. 4.1, a and b).

To further substantiate the specificity of RNA:DNA hybrid staining, some cells were treated with RNase H, an endoribonuclease that specifically degrades RNA in RNA:DNA hybrids (Frank *et al.*, 1994). S9.6 is a monoclonal antibody specific to RNA:DNA hybrids (Boguslawski *et al.*, 1986; Hu *et al.*, 2006). Both PicoGreen and S9.6 signals were decreased, supporting the fact that RNA:DNA hybrids are constitutively present in the tested cell lines (Fig. 4.1). As RNA:DNA hybrids can also form during replication of mitochondrial DNA, cells were stained with the mitochondrial marker COX IV or Mitotracker. 3D rendering of the confocal images showed that the detected RNA:DNA hybrids are localized outside of mitochondria (Fig. 4.1, b and e).

Apart from previous findings that RNA:DNA hybrids can form in the cytosol of retrovirus infected cells (Whitcomb and Hughes, 1992), I found the first instance of extranuclear RNA:DNA hybrids present in many uninfected cell lines, as detected by the antibody S9.6. In addition to the A549 cell line, cell lines of human cancer cell lines derived from colorectal carcinoma (LoVo, HCT 116, HT29), acute monocytic leukemia (THP-1), and normal lung tissue MRC-5 (Fig. 4.2a) were also stained. Some cell lines were also stained by PicoGreen for extranuclear nucleotides, which include RNA:DNA hybrids and may also contain dsDNA (Fig. 4.2b).

In summary, I found that RNA:DNA hybrids are constitutively present in the cytosol of the human cells.

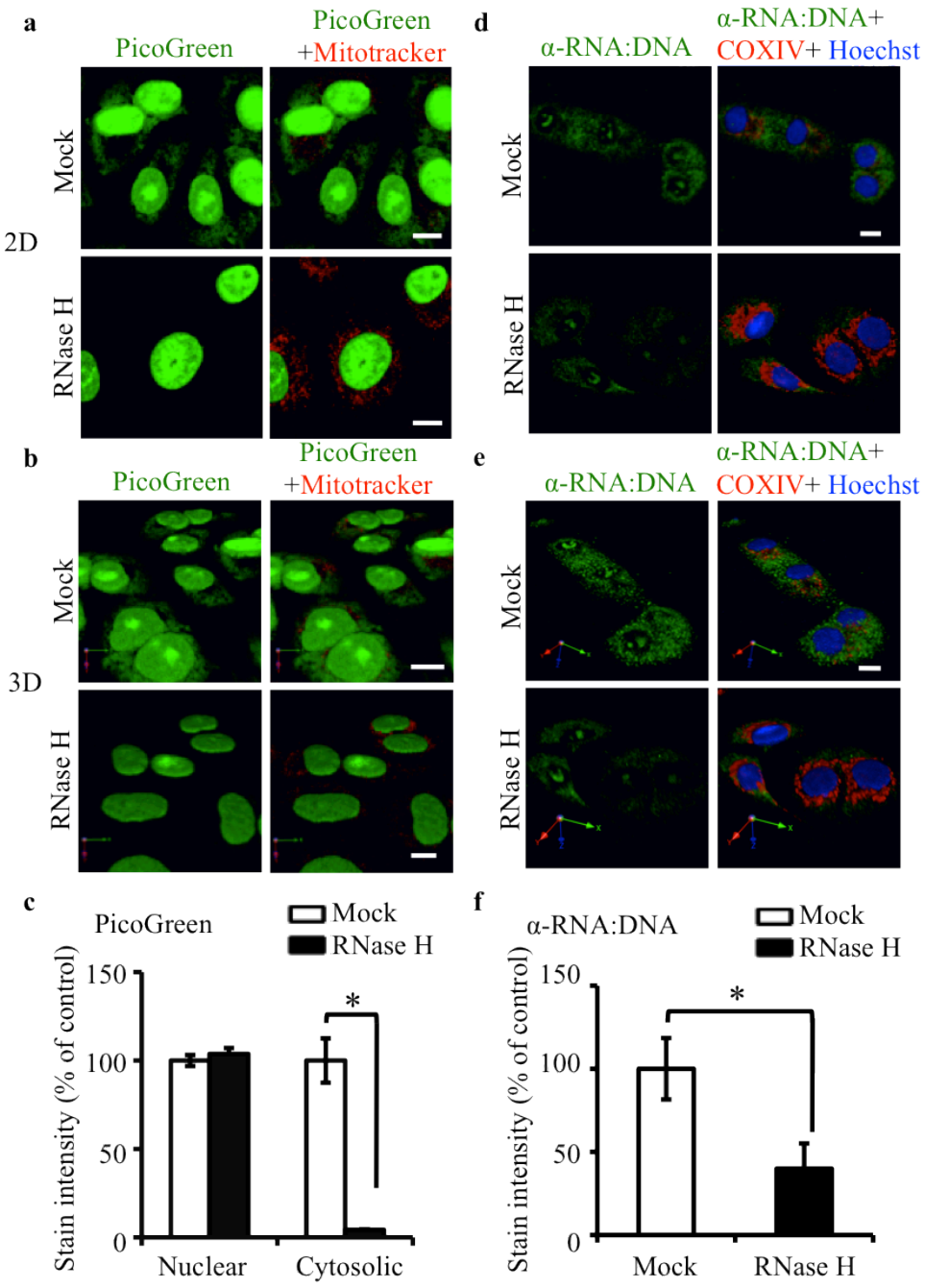


Figure 4.1 Presence of RNA:DNA hybrids in the cytosol of human lung cancer cells. (a) The A549 human lung carcinoma cell line was stained with 10 µl/ml of the vital dsDNA-specific dye PicoGreen (Green) for 1 hr and 100 nM of the mitochondria-specific vital dye MitoTracker (Red) for 30 minutes. Samples shown in lower row were pretreated with 0.5 U/ml of RNase H. (b and c) 3D isosurface rendering (b) and quantification (c) of PicoGreen staining in the nucleus and cytosol of images shown in (a). One-tailed Wilcoxon test was performed. Error bars represent

SEM. * P < 0.05 (d) A549 cells were stained with the RNA:DNA hybrid-specific antibody S9.6 (Green) and the mitochondrial marker COX IV (Red) in the presence of Hoechst (Blue). Cells shown in the lower row were pretreated with 0.5 U/ml of RNase H before staining. (e and f), 3D isosurface rendering (e) and quantification (f) of RNA:DNA hybrid staining of images shown in (d). One-tailed Wilcoxon test was performed. Error bars represent SEM. * P < 0.05.

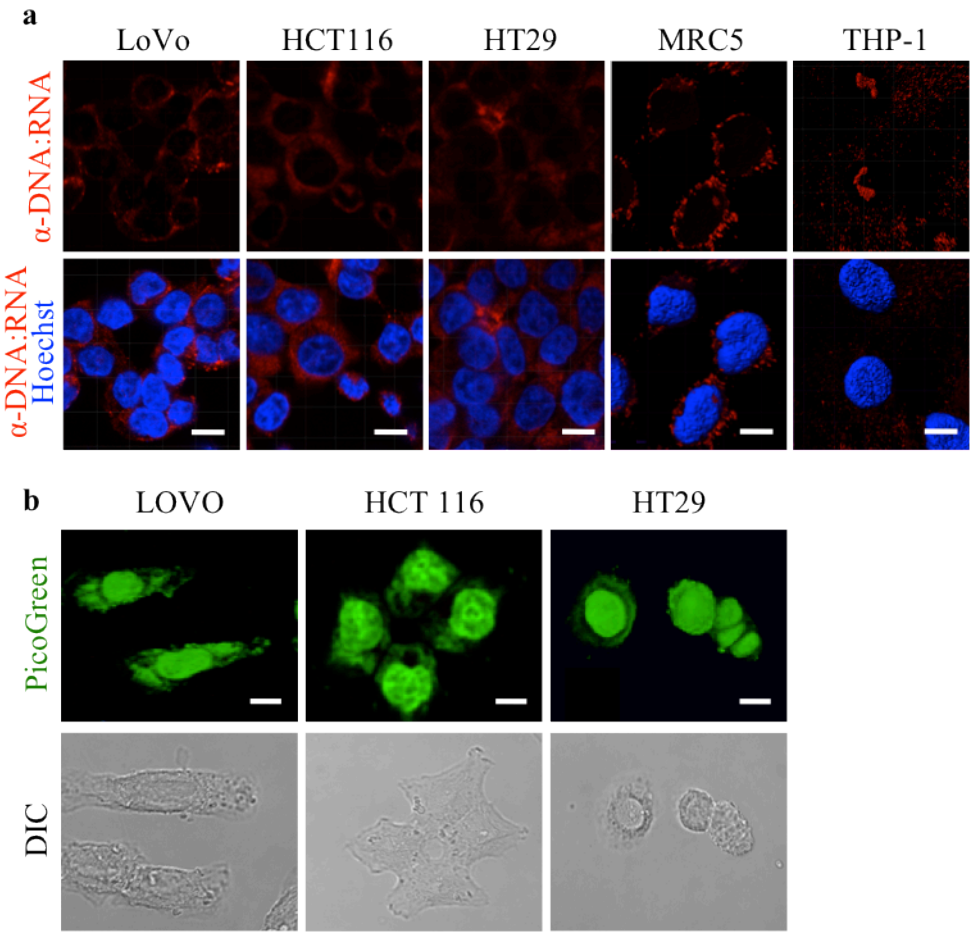


Figure 4.2 Presence of cytosolic RNA:DNA hybrids in human tumor cell lines. (a) The human colorectal carcinoma cell lines LoVo, HCT 116, and HT29, the human acute monocytic leukemia cell line THP-1, and the human normal lung tissue derived cell line MRC-5, were stained for the presence of RNA:DNA hybrids (Red) and Hoechst (Blue). (b) LoVo, HCT116 and HT29 were stained with PicoGreen (Upper row). Bright field images (DIC) of cells are shown in the lower row.

4.2 Specificity of RNA:DNA hybrids

In agreement with the observation in Figure 4.1 that PicoGreen stain is removed with RNase H treatment, S9.6 staining partially co-stained with PicoGreen suggesting that PicoGreen also stains RNA:DNA hybrids in addition to cytosolic dsDNA (Fig. 4.3a). Correspondingly, co-stain of cytosolic RNA:DNA hybrids and dsDNA also showed partial colocalization (Fig. 4.3b), suggesting partial overlap or recognition of these two nucleic acid structures in the cytosol.

Previously, we found that cytosolic dsDNA depends on the DDR in murine cells, and observed the presence of cytosolic dsDNA in murine B-cell lymphoma cells (Lam *et al.*, 2014a). To test if the same cellular response to DNA damage is required for the presence of cytosolic RNA:DNA hybrids in human cells, ATM and ATR, two kinases that initiate the DDR, were inhibited. Strikingly, in contrast to cytosolic dsDNA, inhibition of the DDR had no effect on the presence of RNA:DNA hybrids in the cytosol (Fig. 4.4). Hence, unlike cytosolic dsDNA, RNA:DNA hybrid levels in the cytosol of A549 is not dependent on DDR.

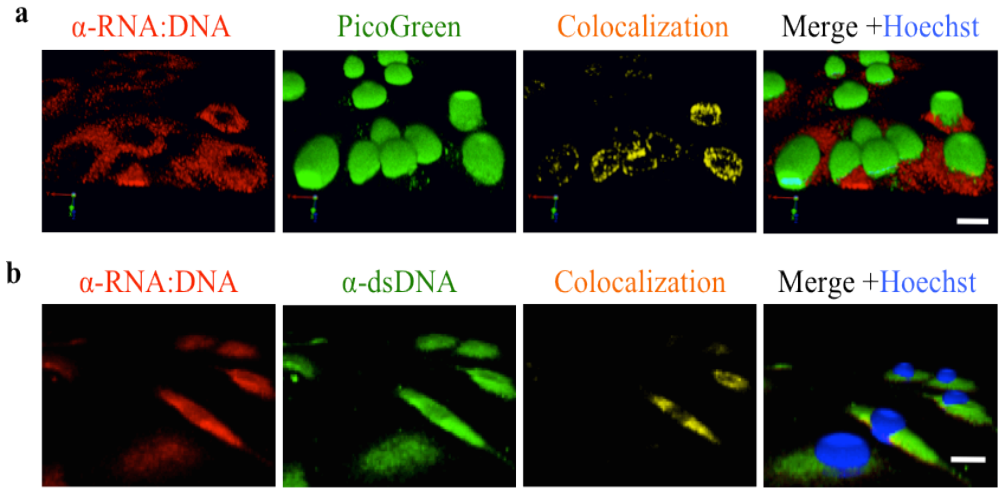


Figure 4.3 Specificity of S9.6 antibody for RNA:DNA hybrids. 3D isosurface rendering of staining of A549 cells with RNA:DNA hybrid-specific antibodies (Red), (a) PicoGreen (Green) or (b) dsDNA antibody (Green), and Hoechst (Blue). Colocalization regions were rendered by Volocity.

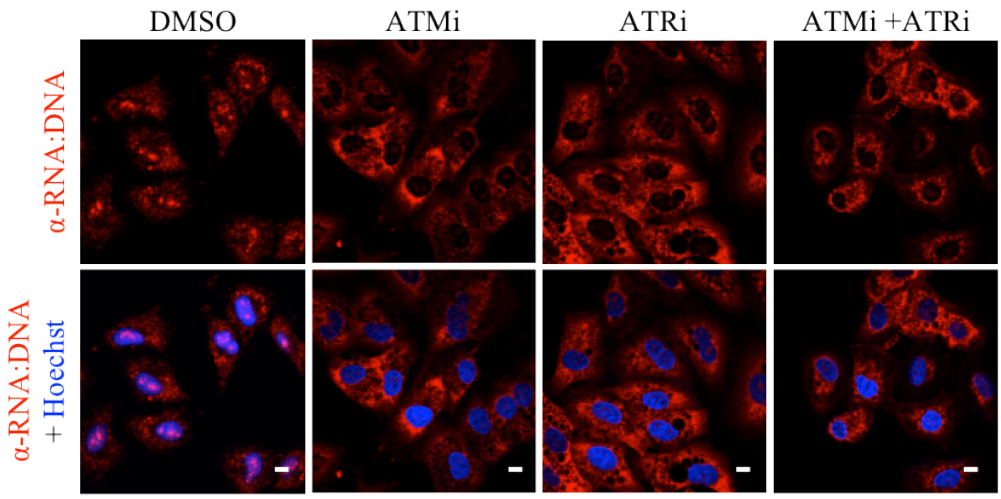


Figure 4.4 Presence of RNA:DNA hybrid does not depend on the DDR. A549 cells were treated with 10 μM of ATM inhibitor (ATMi), or ATR inhibitor (ATRi), or both for 15 hrs. Cells were stained with RNA:DNA hybrids (Red) in the presence of Hoechst (Blue). *Fig. 4.4 kindly contributed by Y.J. Shen.*

4.3 Inhibition of RNA POL III decreases cytosolic RNA:DNA hybrids

RNA:DNA hybrids can occur during transcription of DNA (Shaw and Arya, 2008). To test the role of transcription, A549 cells were treated with RNA POL III inhibitors (Wu *et al.*, 2003). The presence of cytosolic RNA:DNA hybrids was blocked significantly at doses above the IC_{50} (half maximal inhibitory concentration) of ML-60218 for POL III, which is 27 μM in mammalian cells (Fig. 4.5) (Wu *et al.*, 2003). POL III inhibition resulted in a significant decrease in cytosolic PicoGreen staining while not affecting nuclear PicoGreen intensity (Fig. 4.5b), suggesting that PicoGreen also stains large amounts of dsDNA in the nucleus. However, RNA:DNA hybrid antibody staining showed significant decrease of nuclear staining of cytosolic RNA:DNA hybrids at 30 μM of ML-60218 treatment (Fig. 4.5d), suggesting ML-60218 treatment also affects nuclear RNA:DNA hybrids.

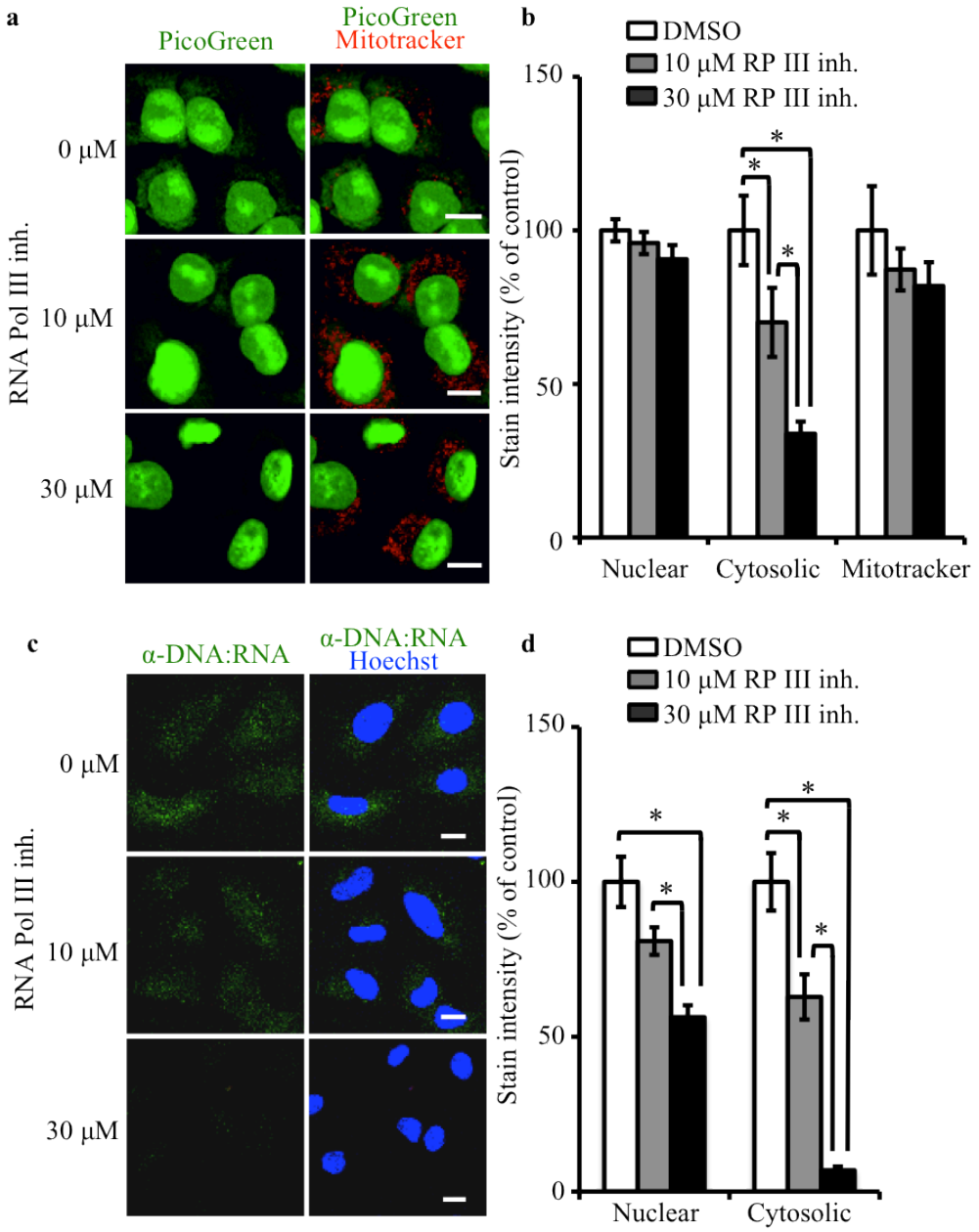


Figure 4.5 The presence of cytosolic RNA:DNA hybrids depends on RNA POL III. (a) A549 cells were treated with the indicated concentration of RNA POL III inhibitor ML-60218 for 3 hrs, before staining with 10 μl/ml PicoGreen (Green) for 1 hr and 100 nM of the mitochondria-specific vital dye MitoTracker (Red) for 30 minutes. (b) Cytosolic and nuclear intensity quantification of images shown in (a). Two-tailed Wilcoxon test was performed. Error bars represent SEM. * $P < 0.05$. (c) A549 cells were treated with indicated concentration of RNA POL III inhibitor ML-60218 for 3 hrs, before staining of cells with RNA:DNA hybrid-specific S9.6 antibodies (Green) and Hoechst (Blue). (d) Cytosolic and nuclear intensity quantification of images shown in (c). Two-tailed Wilcoxon test was performed. Error bars represent SEM. * $P < 0.05$.

To further investigate the role of RNA POL III on RNA:DNA hybrids, control scrambled siRNA or siPOLR3G (#1, #2), were transfected into A549 cells (Fig. 4.6). POLR3G is a subunit of the RNA POL III complex that is involved in catalysis of transcription of DNA into RNA (Wang and Roeder, 1997). Strikingly, siPOLR3G#1 caused a decrease in POLR3G expression detectable by immunofluorescence, which corresponded to diminished levels of cytosolic RNA:DNA hybrids. However, siPOLR3G#2 failed to suppress POLR3G expression and had little effect on RNA:DNA hybrids.

To address if POL III contributes to the generation of RNA:DNA hybrids in the cytosol, we stained A549 cells for POLR3G, a subunit of the POL III complex. In contrast to POL II, which is exclusively localized in the nucleus, a subset of POL III is present in the cytosol, where it might act a putative cytosolic DNA sensor (Chiu *et al.*, 2009). In A549 cells POLR3G was localized in the nucleus and no significant co-staining of POLR3G and RNA:DNA hybrids was observed (Fig. 4.6).

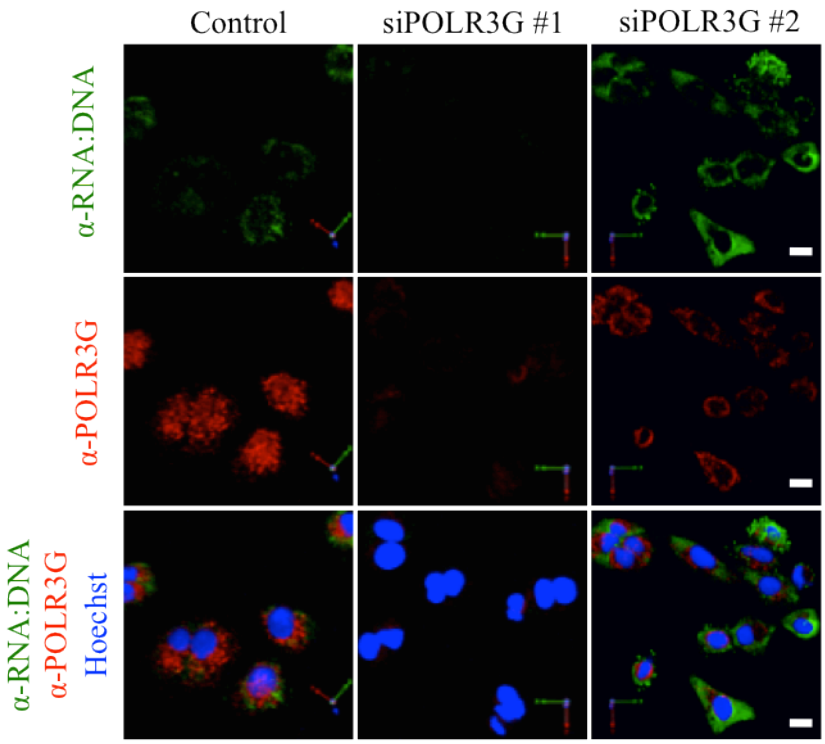


Figure 4.6 siPOLR3G abrogated the presence of cytosolic RNA:DNA hybrids. A549 cells were transfected with 25 nM of control scrambled sequence siRNA, or siRNA against *POLR3G* (siPOLR3G #1, siPOLR3G #2). Two days after transfection, cells were stained for RNA:DNA hybrids (Green), POLR3G (Red), and Hoechst (Blue).

4.4 Presence of RNA:DNA hybrids is independent of DNA damage

RNA:DNA hybrids cause stalling of the replication fork and formation of dsDNA breaks (Aguilera and Garcia-Muse, 2012; Chan *et al.*, 2014; Hamperl and Cimprich, 2014). To test if stalling of replication forks increases the amount of RNA:DNA hybrid in the cytosol, A549 cells were treated with Ara-C and aphidicolin (APH), an inhibitor of DNA polymerases (Pedrali-Noy and Spadari, 1979).

Treatment with Ara-C or APH had no effect on the level of cytosolic RNA:DNA hybrids in A549 cells (Fig. 4.7a, c). Moreover, Ara-C treatment did not increase the co-localization of POLR3G and RNA:DNA hybrids (Fig. 4.7a, b). This suggested that inhibition of DNA replication and replication fork progression is not sufficient to increase the levels of RNA:DNA hybrid in the cytosol. In summary, our data suggest that cytosolic RNA:DNA hybrids are generated by RNA POL III activity in the nucleus, independent of DNA replication.

4.5 RNA:DNA hybrids are present in human lymphoma tissues

To extend the findings of RNA:DNA hybrids present in human cells, and validate these findings in human tissues, we obtained human normal spleen tissue or cancer tissue from a B-cell lymphoma patient. Strikingly, staining of RNA:DNA hybrids showed that while no hybrids were detected in normal spleen tissue, the cancerous spleen tissue had abundant RNA:DNA hybrid staining (Fig. 4.8). This suggested that the presence of RNA:DNA hybrids may be specific towards tumorigenic tissues.

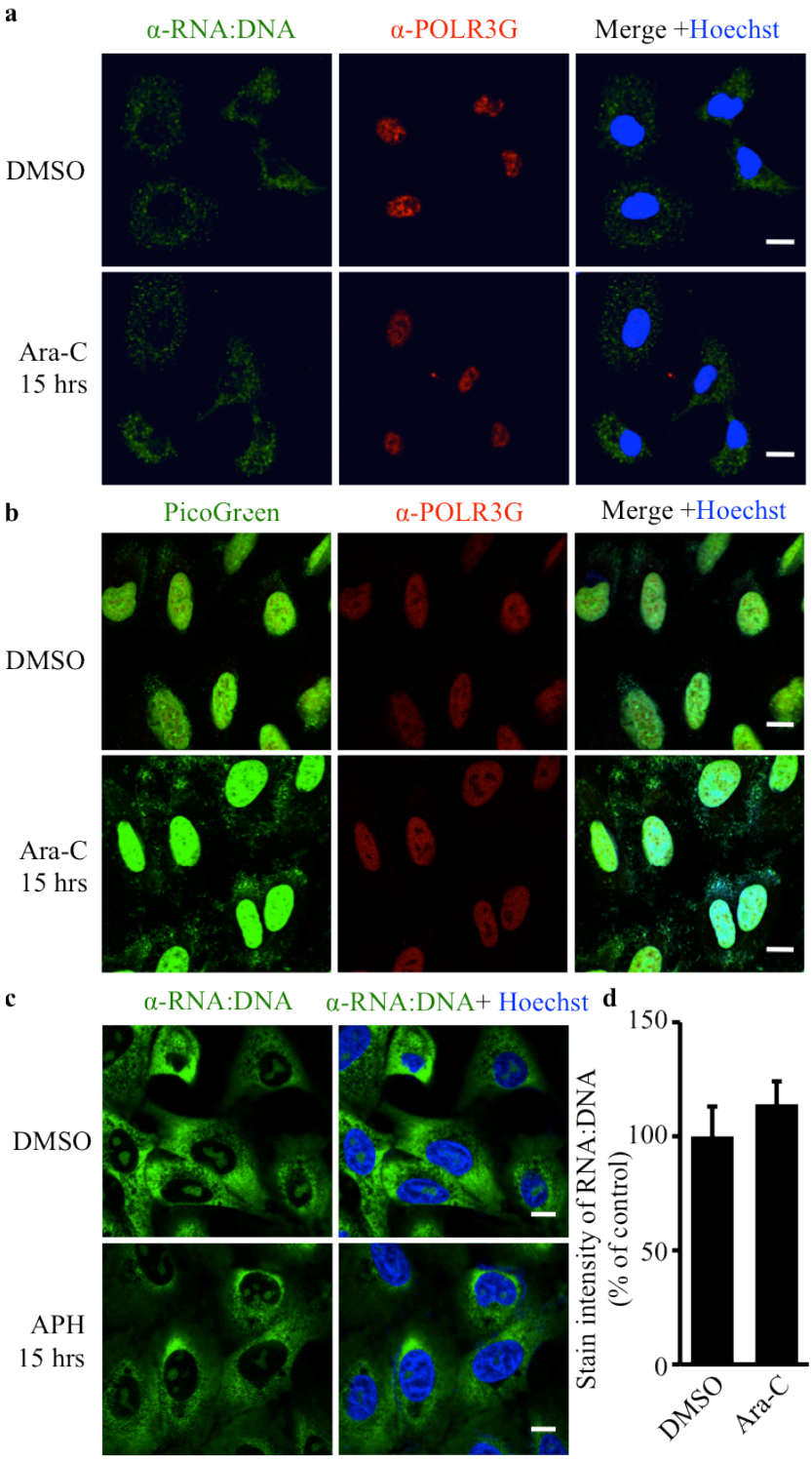


Figure 4.7. Levels of cytosolic RNA:DNA hybrids were not modulated by genotoxic replication inhibitors. (a) A549 cells were treated with 10 μM of the genotoxic DNA replication inhibitor Ara-C for 15 hrs, and stained RNA:DNA hybrids (Green) and POLR3G (Red) in the presence of Hoechst (Blue). (b) A549 cells treated with Ara-C as in (a) and stained with 10 μl/ml PicoGreen (Green), POLR3G-specific antibody (Red), and Hoechst (Blue). (c) A549 cells were treated with 4 μM of aphidicolin (APH), an inhibitor of DNA polymerase for 15 hrs, and stained with RNA:DNA hybrid-specfic antibody (Green) in the presence of Hoechst (Blue). *Fig. 4.7c kindly contributed by M. Khatoo.* (d) Quantification of cytosolic

RNA:DNA hybrid stain in (a). Two-tailed Wilcoxon test was performed. Error bars represent SEM.

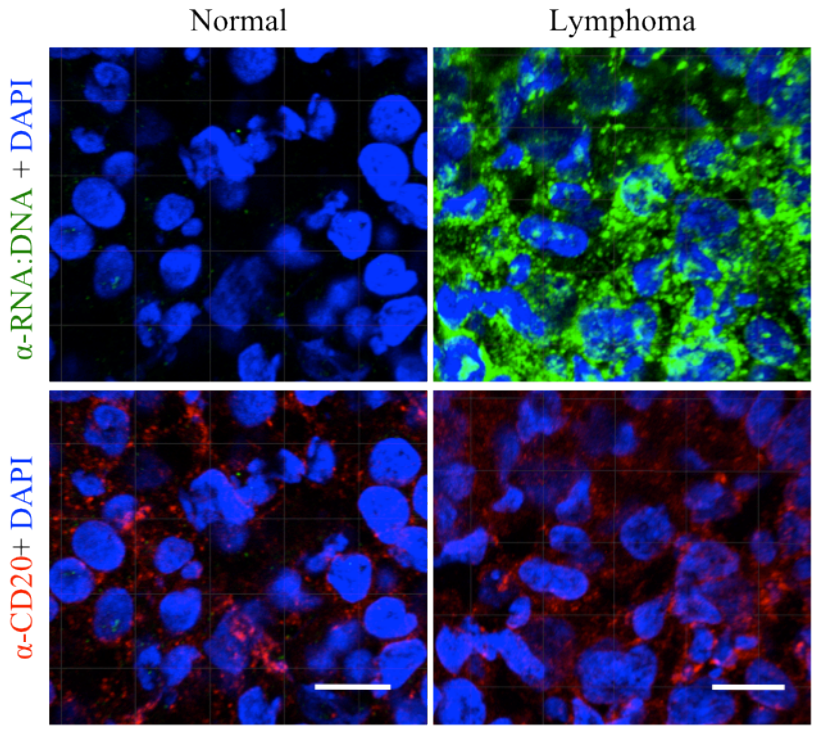


Figure 4.8 RNA:DNA hybrids are present in human lymphoma tissues. Cyrosections of normal human spleen tissue or cancer spleen tissue from a large B cell lymphoma patient were stained for RNA:DNA hybrids (Green), marker of B cells CD20 (Red), and DAPI (Blue).

4.6 Cytosolic RNA:DNA hybrids binds components of the miRNA processing machinery

To determine if RNA:DNA hybrids interact with proteins in the cytosol, we performed immunoprecipitation experiments using RNA:DNA hybrid antibodies on cytosolic extracts of A549 cells. A fraction of the extracts was treated with RNase H before analysis to verify RNA:DNA hybrid-specific binding of proteins. Analysis by mass spectrometry identified DDX5/DDX17 and Argonaute (AGO) 2 as proteins that immunoprecipitated in a RNA:DNA hybrid-dependent manner (Fig. 4.9). These proteins are part of the miRNA processing machinery (Wang and Taniguchi, 2013). Immunoblot analyses of immunoprecipitated proteins were consistent with the mass spectrometry analysis (Fig. 4.10, a and b).

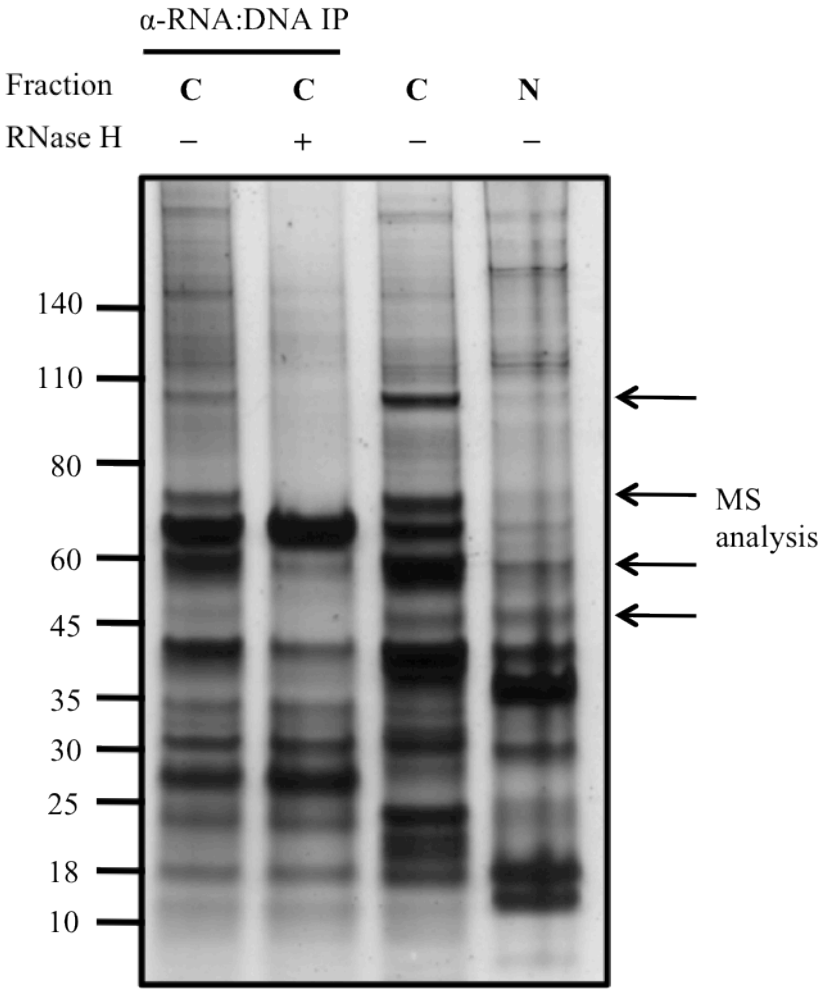


Figure 4.9 Immunoprecipitation of cytosolic RNA:DNA hybrids. Cytosolic fractions of A549 cells were subjected to immunoprecipitation using RNA:DNA hybrid-specific S9.6 antibodies. A part of the cytosolic fraction was pretreated with 0.5 U/ml RNase H. Immunoprecipitated proteins were detected by SDS-PAGE gel electrophoresis and silver staining. Indicated bands were analyzed by mass spectrometry. A portion of pretreated cytosolic fraction and nuclear fraction were loaded on the two rightmost lanes for comparison.

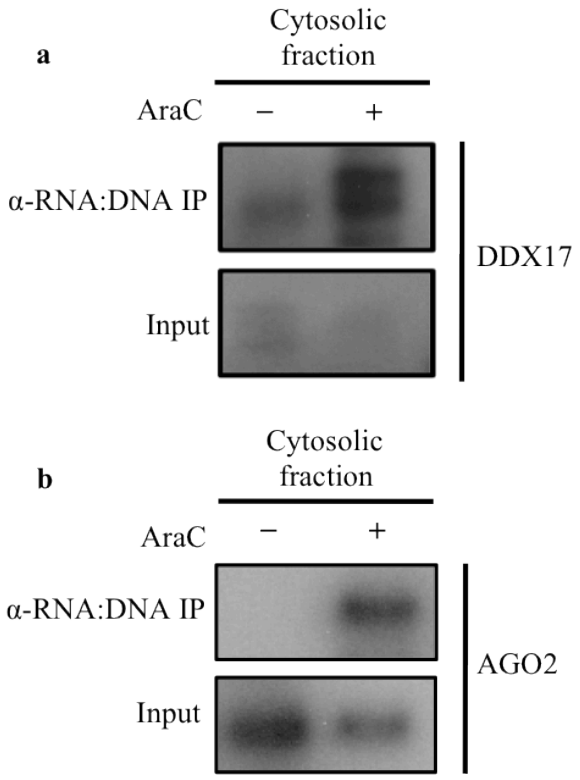


Figure 4.10 Cytosolic RNA:DNA hybrids interact with proteins of the microRNA machinery. A549 cells were treated with DMSO or 10 μM Ara-C for 15 hrs, and harvested for cell fractionation after fixation. Cytosolic fractions were subjected to immunoprecipitation with RNA:DNA-specific S9.6 antibodies. Immunoblot analysis of immunoprecipitated proteins probed with antibodies specific for (a) DDX17 and (b) AGO2.

Chapter 5: RNA POL III affects miRNA expression but not interferon production

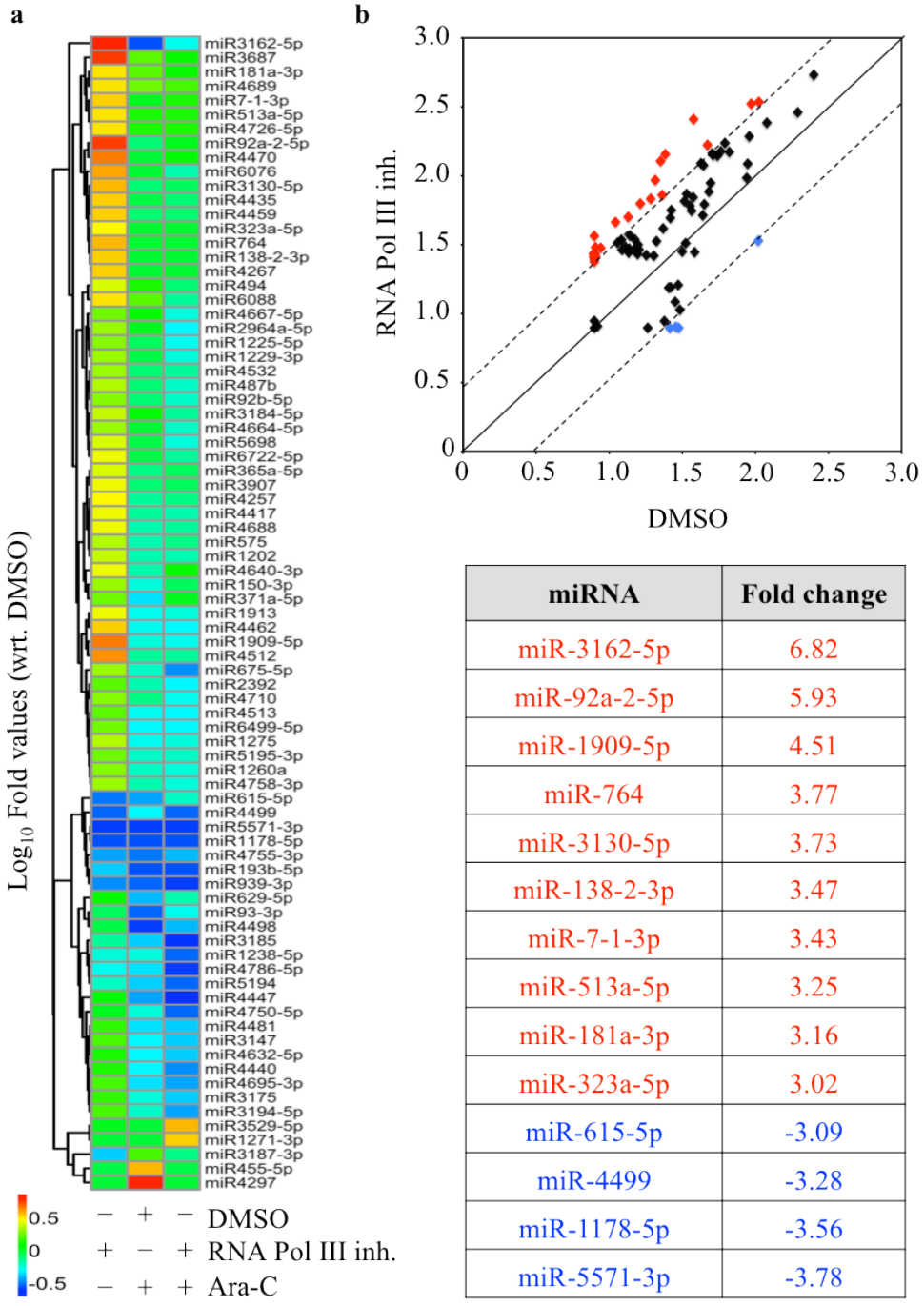
5.1 RNA POL III regulates the expression of specific miRNAs

We sought to gain insights into the RNA POL III-dependent mechanisms leading to the presence of cytosolic RNA:DNA hybrids. Our data support the possibility that RNA POL III-mediated transcription of miRNAs is associated with the generation of RNA:DNA hybrids. As POL III transcribes a subset miRNAs in a cell-type-specific manner (Borchert *et al.*, 2006), we determined the RNA POL III-dependent miRNA expression profile in A549 cells treated with a RNA POL III inhibitor or DMSO, subjected to comprehensive miRNA array analysis. To distinguish RNA POL III effects from RNA:DNA hybrid-induced DNA damage, cells were treated with the genotoxic DNA replication inhibitor Ara-C. A treatment time point of 24 hrs was used to ensure sufficient and stable expression of miRNAs after the RNA POL III inhibitor treatment, as the inhibitor might disrupt cell division if treated for longer periods of time. Results of miRNA expression were normalized across all treatment groups and with respect to DMSO treatment.

A total of 81 differentially expressed miRNA were identified after comparison across the treatment groups (Fig. 5.1a). Strikingly, treatment of cells with POL III inhibitor resulted in significant downregulation of only four miRNAs: miR-615-5p, miR-1178-5p, miR-4499, and miR-5571-3p (Fig. 5.1, a and b). The expression of miR-615-5p, miR-1178-5p, and miR-5571-3p was also decreased after Ara-C treatment, which suggested that these miRNAs were regulated by RNA:DNA hybrid-induced DNA damage. In contrast, miR-4499 is likely to be directly transcribed by POL III as Ara-C had no effect on its expression. Surprisingly, the expression of ten miRNAs was upregulated after treatment with the POL III inhibitor ML-60218, but not Ara-C (Fig. 5.1, a and b). In summary, our data indicate that POL III regulates the expression of only a number of miRNAs in A549 cells. Hence, it is unlikely that cytosolic RNA:DNA hybrids consists of POL III-transcribed microRNAs in A549 cells.

To test if expression changes in transcripts targeted by POL III-modulated miRNAs could account for the presence of cytosolic RNA:DNA, a miRNA target prediction and pathway analysis was performed using the DIANA miRPath software (http://www.microrna.gr/miRPathv2.) (Vlachos *et al.*, 2012). Among the top ranked pathways, RNA transport and RNA surveillance pathways were identified to contain predicted genes that are targeted by two or more ML-60218-induced miRNAs (red boxes), while other transcripts are potentially targeted by a single miRNA (yellow boxes) (Fig. 5.2). Hence, RNA transport or stability may contribute to the presence of RNA:DNA hybrids.

To test the predictability of the DIANA-miRPath software, three genes *KPNB1*, *XPO1*, and *NUP153* were selected for mRNA expression detection after RNA POL III inhibition. Gene expression of *XPO1* and *NUP153* were found to be downregulated by 1.6-fold and 3.3-fold respectively, while *KPNB1* was not significantly downregulated (Fig. 5.3), after POL III inhibition as compared to DMSO.



miRNA	Fold change
miR-3162-5p	6.82
miR-92a-2-5p	5.93
miR-1909-5p	4.51
miR-764	3.77
miR-3130-5p	3.73
miR-138-2-3p	3.47
miR-7-1-3p	3.43
miR-513a-5p	3.25
miR-181a-3p	3.16
miR-323a-5p	3.02
miR-615-5p	-3.09
miR-4499	-3.28
miR-1178-5p	-3.56
miR-5571-3p	-3.78

Figure 5.1 Dysregulation of microRNAs levels in response to RNA POL III inhibition. (a) A549 cells were treated with 10 μM of the RNA POL III inhibitor ML-60218 (RPIII inh.) or DMSO for 24 hrs. Some cells were further treated with Ara-C for 15 hrs. Heat map of global miRNA expression profile from total RNA extracted after treatment is shown. All expression profiles were quantile-normalized and then fold values with reference to control (DMSO) were used for plotting this heatmap. 81 probes had at least 3-fold differential expression in any pair of conditions and were pre-selected for this plot. (b) Scatter-plot of miRNA expression profiles between control (DMSO) and ML-6028-treated samples. Decision surface was plotted for at least three-fold changes to or from control. Highly upregulated and downregulated miRNAs after RPIII inhibitor treatment were identified in the table (Lower columns).

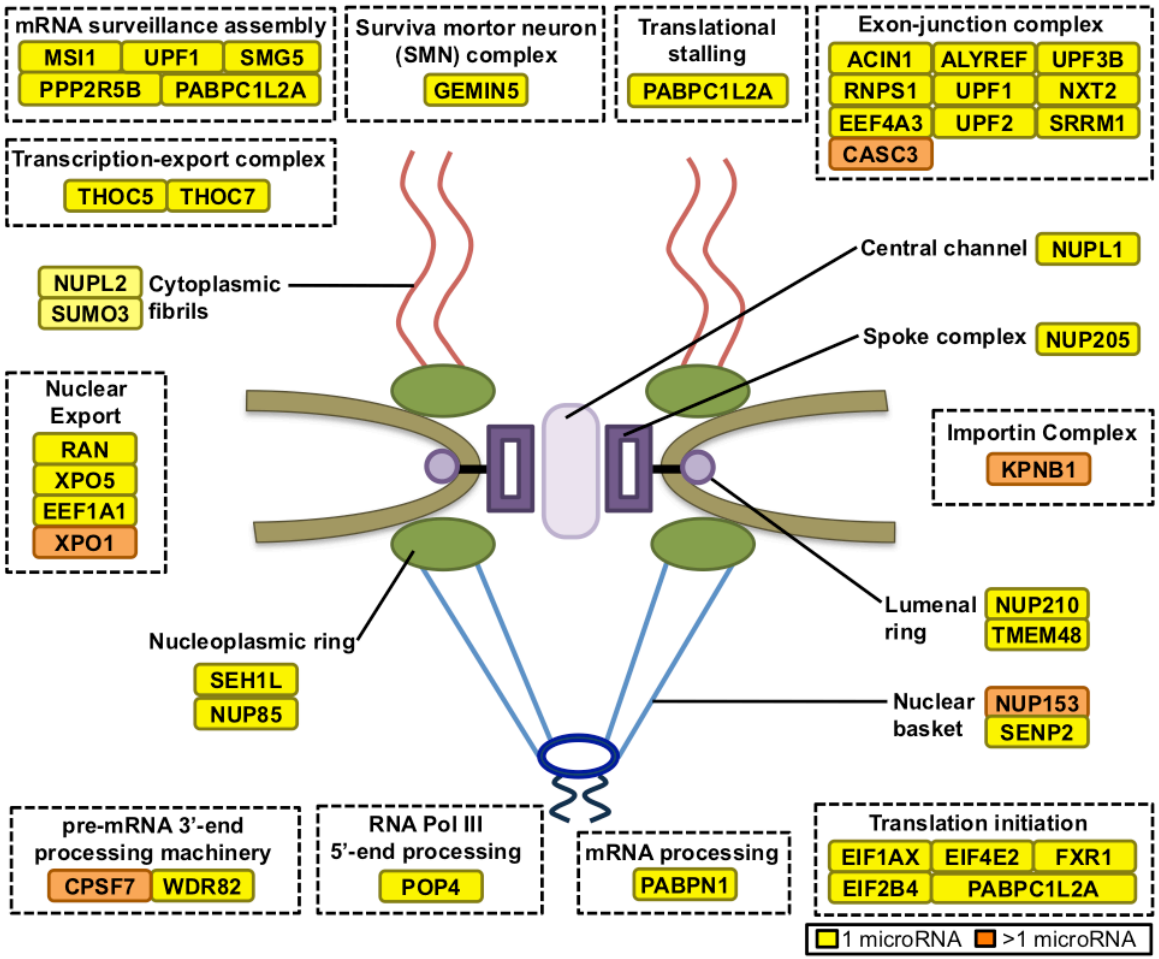


Figure 5.2 RNA transport and mRNA surveillance pathways are potential targets of RNA polymerase III modulated miRNAs.

Figure 5.2 RNA transport and mRNA surveillance pathways are potentially regulated by RNA POL III modulated miRNAs. List of predicted miRNAs regulated by POL III were analyzed for potential gene targets by using online resource DIANA-miRPath. Yellow boxes indicate genes that are regulated by one miRNA in response to POL III inhibition. The red boxes indicate potential target genes regulated by more than one miRNA upon POL III inhibition.

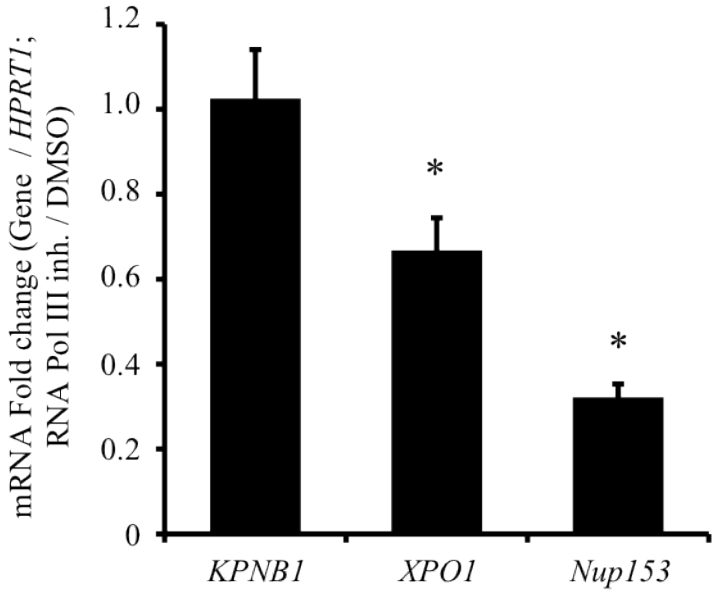


Figure 5.3 RNA POL III inhibition decreased mRNA expression of POL III-regulated miRNA targets. mRNA expression levels of three potential genes, each targeted by more than one miRNA. Total RNA was extracted from A549 cells after treatment with 10 μM of the RNA POL III inhibitor ML-60218 (RPIII inh.) or DMSO for 24 hrs, and subjected to real time PCR analysis. Normalization was done with respect to *HPRT1* housekeeping gene, and further compared against DMSO-treated cells. One-tailed Wilcoxon test was performed. Error bars represent SEM. * $P < 0.05$.

5.2 Interferon-beta levels are refractory to siRNA against RNA POL III

Another possibility of RNA POL III-directed effects of RNA:DNA hybrids is the induction of interferons for innate immune sensing. Previously, transfection of poly(dA:dT) was sensed by the cytosolic DNA sensor RNA POL III, and induced IFN-β reporter activity (Ablasser *et al.*, 2009). Transfected RNA:DNA hybrids of 60 base-pairs were also able to induce cytokine and interferon production (Rigby *et al.*, 2014). Since the presence of cytosolic RNA:DNA hybrids were dependent on RNA POL III activity, we blocked RNA POL III activity to determine if RNA:DNA hybrid presence could affect interferon expression in tumor cells. Cells were transfected with control scrambled siRNA, or siPOLR3G (#1 and #2), for 48 hrs, before analyzed for POLR3G knockdown and *IFNb* mRNA expression. Although knockdown of POLR3G, a functional subunit of RNA POL III, was achieved, *IFNb* expression was not diminished in all knockdowns suggesting that siRNA of POLR3G was insufficient to dampen *IFNb* expression (Fig. 5.4).

To examine chemical inhibition of RNA Pol III on *IFNb* expression, cells were treated with RNA POL III inhibitor ML-60218 before quantitative analysis of *IFNb*. Low concentrations of ML-60218 at 10 μM did not decrease *IFNb* expression (Fig. 5.5). Interestingly, at 30 μM of ML-60218 for 3 hrs, while RNA:DNA hybrids were shown to disappear (Fig. 4.5), no observable decrease in *IFNb* was detected. At 6 hrs after treatment with 30 μM ML-60218, no significant fold-change decrease of *IFNb* expression was detected, suggesting RNA POL III inhibition did not affect IFNβ levels that might be regulated by RNA:DNA sensing.

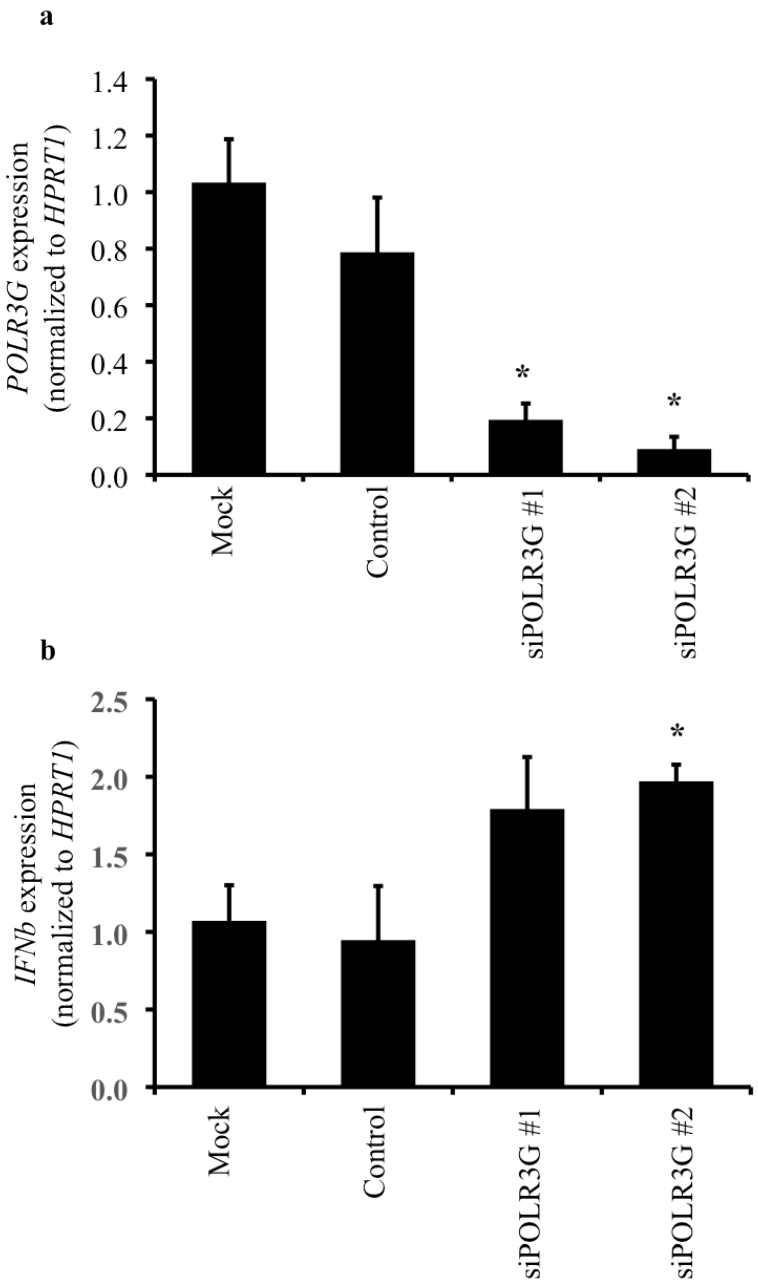


Figure 5.4 siPOLR3G does not regulate *IFNb* levels. Interferon expression of A549 cells in response to siPOLR3G. A549 cells were transfected with 25 nM of control scrambled sequence siRNA, or siRNA against *POLR3G* (siPOLR3G #1, siPOLR3G #2). 48 hrs later RNA was extracted to measure mRNA expression of (a) *POLR3G* and (b) *IFNb* with respect to *HPRT1* as a normalization control. One-tailed and two-tailed Wilcoxon test was performed for (a) and (b) respectively. Error bars represent SEM. * $P < 0.05$.

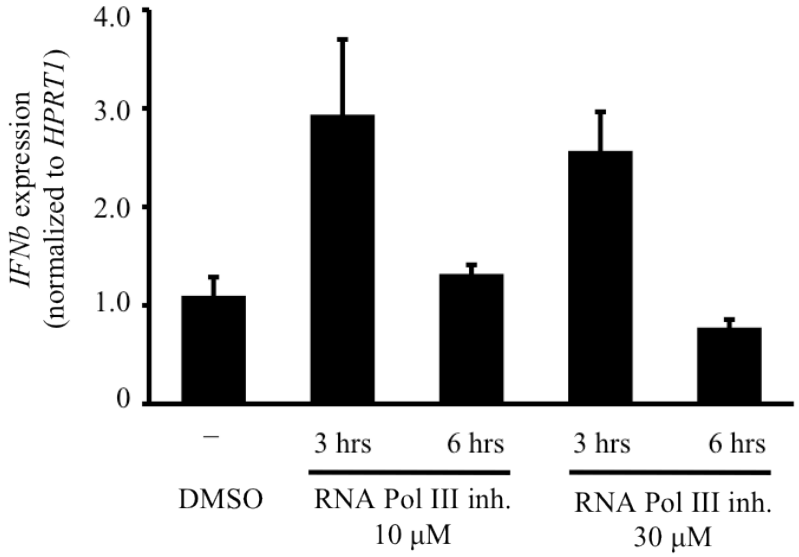


Figure 5.5 Interferon response was not affected at low levels of chemical RNA POL III inhibition. Interferon expression of A549 cells in response to RNA POL III inhibition by ML-60218. Cells were either treated with DMSO, or 10 μM, or 30 μM of ML-60218, for increasing periods of time (3, 6 hrs). RNA was extracted to measure mRNA expression of *POLR3G* and *IFNb* with respect to *HPRT1* as a normalization control. Two-tailed Wilcoxon test was performed. Error bars represent SEM.

Chapter 6: *Tnfa* deficiency reduces survival of Eμ-*Myc* mice

6. *Tnfa*-deficient Eμ-*Myc* mice

The occurrence of cytosolic DNA in tumor cells may lead to the activation of the cytosolic DNA sensor pathways, which induces the expression of interferons and other pro-inflammatory cytokines (Lam *et al.*, 2014a). Using Eμ-*Myc* mice, a mouse model of spontaneous B cell lymphoma, we found that mice deficient in potential cytosolic DNA sensor *Zbp1/Dai* had higher survival when compared to $Zbp1^{+/+}$Eμ-*Myc* mice (Lam et al., manuscript submitted). We showed that cytosolic DNA accumulates in the B cell tumors of Eμ-*Myc* mice, suggesting that dsDNA increases with tumorigenesis. This possibility of dsDNA as a sensor of initial tumor development for immune system early recognition, led us to investigate how the immune system affects tumor load progression in these mice.

6.1 Decreased survival rate of *Tnfa*-deficient Eμ-*Myc* mice

TBK1 acts as a central transducer node in many signaling pathways of immunity (Helgason *et al.*, 2013; Zhao, 2013). It integrates upstream pathogen detection, inflammation, or Ras-induced oncogene signals, and depending on adaptor recruitment, modulates functions towards immune response, inflammation, and processes such as proliferation and autophagy (Helgason *et al.*, 2013). To determine if the STING/TBK/IRF3 pathway for interferon signaling (Miyahira *et al.*, 2009; Ishikawa and Barber, 2011) is activated during accumulation of cytosolic dsDNA in tumor cells, I crossed Eμ-*Myc* mice with *Tnfa/Tbk1*-deficient mice. *Tbk1*-deficient mice are embryonic lethal, but can be rescued by *Tnfa*-deficiency (Perry *et al.*, 2004). Wild type littermate controls were also included to minimize differences in interpretation of data that might be due to the 129-mixed background of $Tnf^{-/-}Tbk1^{-/-}$ mice (Bygrave *et al.*, 2004; Ishii *et al.*, 2006). To exclude the possibility of TNF playing a role in the anti-tumor effect by TBK1, I also crossed *Tnfa*-deficient mice with Eμ-*Myc* mice for comparison. TNF is a known proinflammatory cytokine that plays a role in the antitumor response (Carswell *et al.*, 1975). Thus knockout of TNF

is hypothesized to reduce the antitumor response in Eμ-Myc mice, leading to lower survival due to persistently high tumor load.

Tnfa$^{+/-}$*Tbk1*$^{+/-}$Eμ-*Myc* mice have a shorter survival period as compared to *Tnfa*$^{+/+}$*Tbk1*$^{+/+}$Eμ-*Myc* mice (Fig. 6.1a). Strikingly, *Tnfa*$^{-/-}$ *Tbk1*$^{-/-}$Eμ-*Myc* mice also had shorter survival periods, following a similar survival curve as *Tnfa*$^{+/-}$*Tbk1*$^{+/-}$Eμ-*Myc* mice. Furthermore, *Tnfa*$^{+/-}$Eμ-*Myc* mice also had reduced survival (Fig. 6.1b). Similar to a previous report that *Tnf* heterozygosity affected the levels of TNF by 20 to 100-fold, and compromised the immune response of mice (Amiot *et al.*, 1997), loss of one functional allele of *Tnfa* was sufficient to observe a phenotypic change in Eμ-*Myc* survival. This suggests that TNF acts as a significant pro-inflammatory cytokine and actively contributes to the sensing of tumor cells by the immune system, irrespective of TBK1 function.

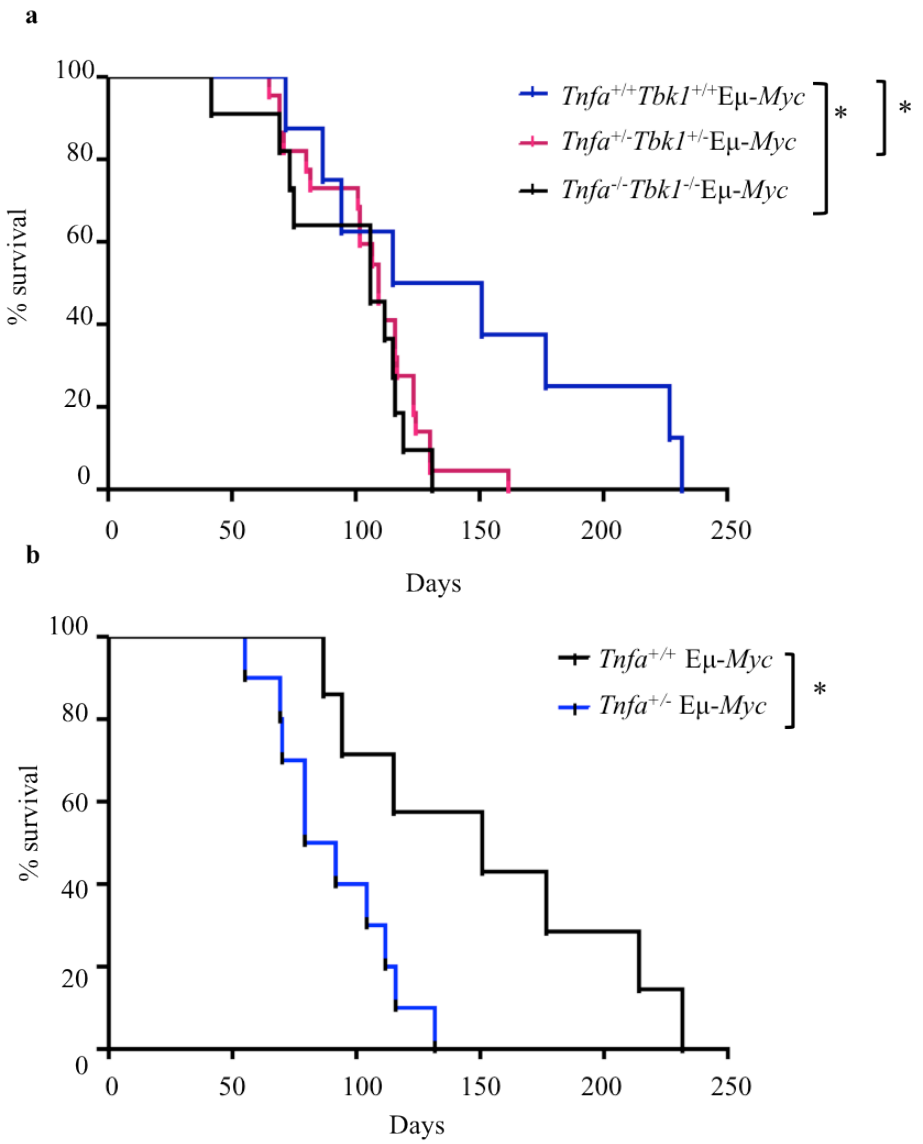


Figure 6.1 Survival curve of Eμ-*Myc*;*Tnfa/Tbk1* double-knockout and Eμ-*Myc*;*Tnfa* mice. (a) The survival of mice (from the date of birth until mortality) is compared between control *Tnfa*$^{+/+}$*Tbk1*$^{+/+}$Eμ-*Myc* (blue curve; n=8), *Tnfa*$^{+/-}$*Tbk1*$^{+/-}$Eμ-*Myc* (red curve; n=15), and *Tnfa*$^{-/-}$*Tbk1*$^{-/-}$Eμ-*Myc* knockout mice (black curve; n=11). * $P < 0.05$ by Log-Rank test or by Gehan-Breslow-Wilcoxon Test. (b) Survival curve of Eμ-*Myc* mice deficient in *Tnfa*. *TNF*$^{+/-}$;Eμ-*Myc* mice (Blue line, n=10) exhibit decreased survival compared to *TNF*$^{+/+}$;Eμ-*Myc* mice (Black line, n=7). * $P < 0.05$ by Log-Rank test or by Gehan-Breslow-Wilcoxon Test.

6.2 Analysis of *Tnfa*-deficient Eμ-*Myc* mice tumor load

Eμ-*Myc* mice demonstrate a spontaneous drop in tumor load from day 40 to day 60, as assessed by the percentage of $B220^{low}$ tumorigenic B cells in peripheral blood (Croxford *et al.*, 2013). This is dependent upon T and NK cells recognition of tumor cells. To determine if this antitumor regression phase in Eμ-*Myc* mice is a result of activation of DNA-sensing mechanisms via TBK1, tumor loads of *Tnfa*$^{-/-}$ *Tbk1*$^{-/-}$Eμ-*Myc* mice and *Tnfa*$^{+/-}$Eμ-*Myc* mice were characterized. While *Tnfa*$^{-/-}$*Tbk1*$^{-/-}$ Eμ-*Myc* and *Tnfa*$^{+/-}$Eμ-*Myc* mice exhibited significant tumor regression of tumor load (Fig. 6.2), overall tumor load for *Tnfa*$^{+/-}$Eμ-*Myc* mice was still higher than Eμ-*Myc* mice at all time periods from day 21-60. Further, *Tnfa*$^{-/-}$*Tbk1*$^{-/-}$Eμ-*Myc* tumor load levels remained low (less than 60%) throughout regression, while *Tnfa*$^{+/-}$Eμ-*Myc* tumor load levels were elevated (more than 60%) up to day 50 of mice tumor load progression. This suggested that mice with reduced levels of TNF lacked sufficient proinflammatory TNF to enhance tumor cell killing, corroborating previous results from the survival curve of *Tnfa*$^{+/-}$Eμ-*Myc* mice (Fig. 6.1). Interestingly, the high tumor load was diminished in *Tnfa*$^{-/-}$*Tbk1*$^{-/-}$Eμ-*Myc* mice, suggesting that compensatory immune sensing mechanisms might replace the TBK1-signalling pathway that produces interferon and cytokines, when TBK1 is not expressed.

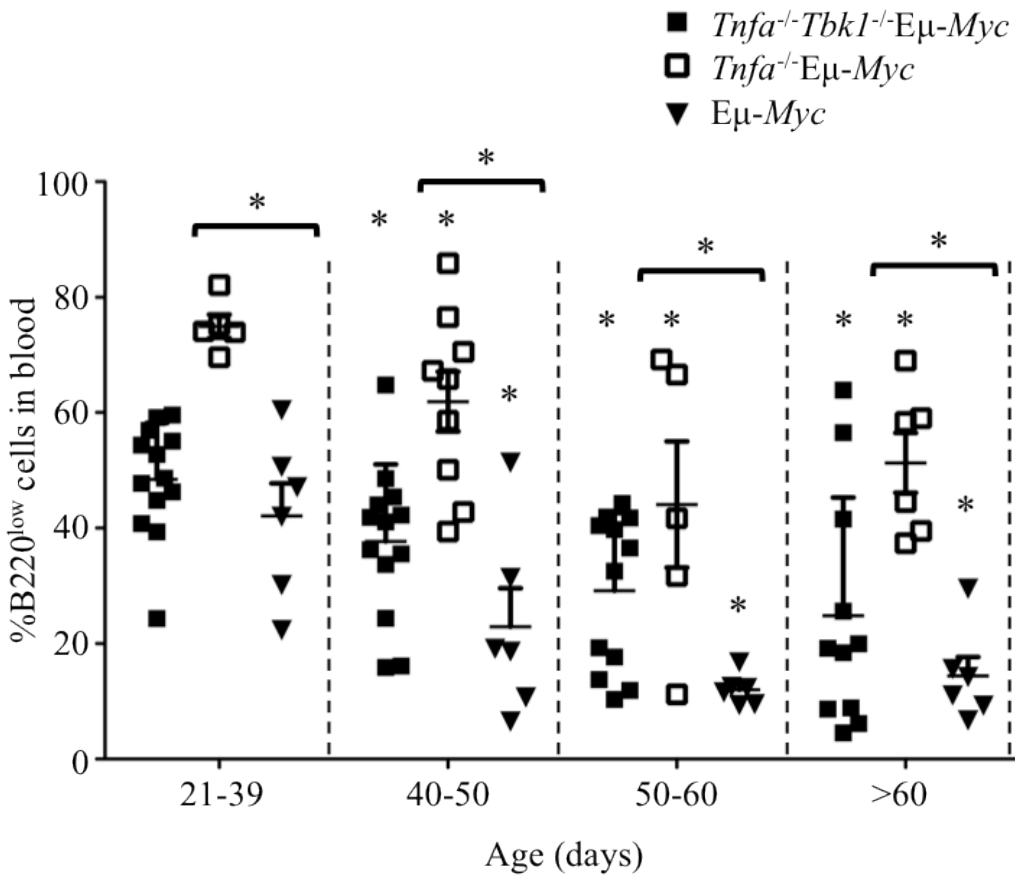


Figure 6.2 Tumor load analysis of Eμ-*Myc*;*Tnfa/Tbk1* double-knockout, Eμ-*Myc*;*Tnfa*, and Eμ-*Myc* mice. The percentage of tumor cells in peripheral blood of *Tnfa*$^{-/-}$*Tbk1*$^{-/-}$Eμ-*Myc* knockout mice (black squares), or *Tnfa*$^{+/-}$Eμ-*Myc* mice (blank squares), or Eμ-*Myc* mice (black triangles) was determined over a period of day 20 to day 65. Data analysed by non-parametric Mann-Whitney Wilcoxon rank-sum test. Error bars represent SEM. * over tumor loads represent $P < 0.05$ compared to corresponding tumor load at D21-39. * over drawn lines represent $P < 0.05$ corresponding to the two columns that the line demarcates.

Chapter 7: Discussion and future perspectives

7.1 Summary of key findings

In this study, the existence of endogenous nucleic acids in human tumor cells was investigated. dsDNA, as well as RNA:DNA hybrids, was found to accumulate in the cytosol of a variety of uninfected human cells, including cancer cells. Cytosolic dsDNA did not wholly colocalize with histones or other DNA binding proteins. DNA damage from exogenous sources triggers the DDR in response to DSB and ATM/ATR dependent increase in cytosolic dsDNA that binds to DDX17.

Conversely, RNA:DNA hybrids are constitutively present in cells and are not dependent upon DNA damage or DDR. RNA:DNA hybrid accumulation depends on RNA POL III, and RNA:DNA hybrid-binding proteins DDX17 and AGO2 were determined through co-immunoprecipitation. These proteins were also involved in miRNA processing machinery, providing a possibility of miRNA regulation via RNA:DNA hybrid accumulation. RNA POL III-specific miRNAs were identified, and target genes of miRNAs included genes coding for proteins in the nuclear pore complex and translation, and transcription machinery. Some of these targets were downregulated in a similar manner after RNA POL III treatment by real-time PCR, suggestive of the cogency of gene target prediction from POL III-specific miRNAs.

To investigate the role of DNA damage on tumorigenesis, I bred $TNF^{-/-}TBK^{-/-}$ mice to Eμ-*Myc* mice. $TNF^{-/-}$Eμ-*Myc* mice alone displayed decreased survival and high tumor loads through the regression phase, revealing TNF-α to be an essential proinflammatory cytokine for tumor cell killing. The importance of DNA damage in tumor cells, and the subsequent cytokine response in Eμ-*Myc* malignancy thus requires TNF-α as a mediator of the immune cell response.

We proposed a model of RNA:DNA hybrid generation and regulation in human cells based on our findings (Fig. 7.1).

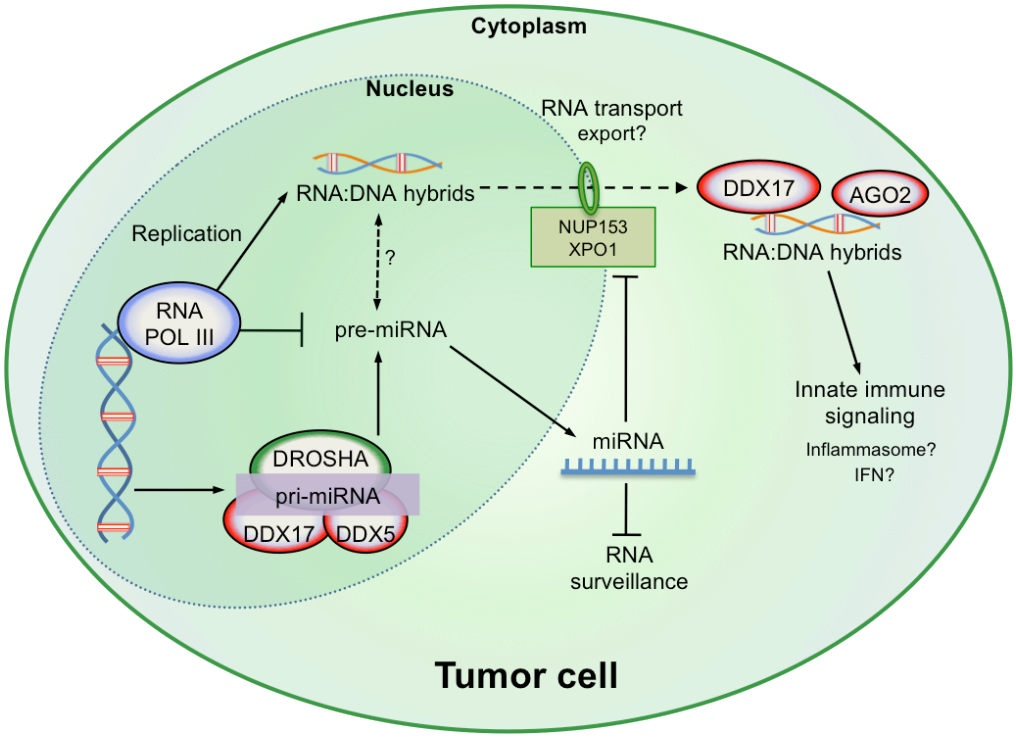


Figure 7.1 Proposed model of RNA:DNA hybrids and RNA POL III.
In tumor cells, constant rapid replication of certain genome sequences by RNA POL III result in RNA:DNA hybrids that localize in the cytosol of cells, binding to DDX17 and AGO2. These structures may be sensed for immune signaling through interferons or the inflammasome. RNA POL III also regulates miRNA profile expression by inhibiting expression of some miRNAs that target pathways in RNA transport and RNA surveillance. RNA:DNA hybrids may also be generated via miRNA modulation.

7.2 dsDNA and RNA:DNA hybrids are derived from two distinct pathways

The DNA damage pathway has been long associated with interferons and the activation of the immune response. Genotoxic stress is a strong precursor of the DDR, which is also triggered in infections and cancers. These mechanisms allow a distinct regulatory pathway by which the body could discriminate against diseased or inflamed tissues or cells. The study of DNA sensors have expanded the field of innate immune sensing pathways; however current work in the field focuses on DNA sensors against exogenous DAMPs such as viral DNA and RNA present during infections.

Here, my findings show that cytosolic nucleic acids are present constitutively in human cancer cell lines, providing another possibility by which DAMPs could be generated to affect cellular processes and the immune response. Cytosolic dsDNA is found to accumulate in response to DNA damage and this phenomenon is dependent on the ATM/ATR DDR pathway. However, the presence of cytosolic RNA:DNA hybrids appears to be independent of the DDR as treatment with the genotoxic agents did not increase the levels of cytosolic RNA:DNA hybrids, and blocking of ATM and ATR had no effect on the amounts of RNA:DNA hybrids in the cytosol. This prominent difference in the regulation of these nucleic acids suggests that derivation of nucleic acids may not be as closely associated, such that the sequences or genomic regions from which dsDNA is created are not similar to regions where RNA:DNA hybrids are derived. Cytosolic dsDNA accumulation is DNA damage-driven, suggesting a response mechanism that serves to repair the cell, and created dsDNA is due to severe lesion damage. In many of the cell lines and tumor tissues I studied, both dsDNA and RNA:DNA hybrids were constitutively present, suggesting that DDR activation was active in these cells. It would be interesting to test if primary cells have cytosolic RNA:DNA hybrids but absence of cytosolic dsDNA, and to determine the severity of DNA damage that would result in accumulation of dsDNA.

Conversely, if certain cells are abundant in either type of nucleic acid in the cytosol, the differences in cell damage state or gene expression might explain this discrepancy in accumulation.

One of the foremost possibilities for the source of cytosolic DNA is the release of mitochondrial DNA (mtDNA) into the cytosol after DNA damage. Oxidized mtDNA from the damaged mitochondria would activate the NLRP3 inflammasome to trigger interferon and cytokine production (Shimada *et al.*, 2012). However, we stained dsDNA with anti-8-oxo, a protein that targets the 8-oxo protein and ROS damaged DNA, and found no colocalization with dsDNA, suggesting that cytosolic dsDNA is not derived from damaged mitochondrial DNA. Further, Mitotracker stain did not co-stain with PicoGreen, nor did COX IV co-stain with either dsDNA or RNA:DNA hybrids, further corroborating the distinct character of these cytosolic nucleic acids as opposed to mitochondrial DNA.

Several lines of evidence point to these nucleic acids being derived from the nucleus. Sequencing of cytosolic DNA showed that many its sequences were derived from the genome, and no mitochondrial sequences were detected (Shen et al., manuscript submitted). Further, inhibition of RNA POL III prevented accumulation of cytosolic RNA:DNA hybrids while hybrids in the nucleus remained steady. Co-stain of these nucleic acids also did not completely colocalize with Mitotracker or COX IV, eliminating the possibility of mitochondrial DNA. Further, treatment of Plasmocin and PenStrep ensured that infection was prevented as bacterial and viruses contribute to cytosolic DNA.

Interestingly, we found that RNA POL III regulates RNA:DNA hybrid accumulation. RNA POL III functions as a transcription related enzyme, recruited as a cell undergoes replication, where it is required to supply tRNAs, along with transcription of ribosomal 5S rRNA and other non-coding RNAs (White, 2011). As RNA POL III binds basal transcription machinery within the nucleus, there is a possibility that hybrids are derived from transcription stalling of the transcription

complex. Recent studies hypothesize possible mechanisms for R-loop induced instability as a source of DSB (Hamperl and Cimprich, 2014). Thus it is possible that presence of RNA:DNA hybrids may precede accumulation of dsDNA that occurs with extensive DNA damage. It will be interesting to investigate the mechanisms by which these two types of nucleic acids could interplay with each other.

Ara-C, the DNA damage agent that resulted in increased cytosolic dsDNA, is normally administered for chemotherapy, particularly in B cell lymphoma patients. It is metabolized into ara-CDP and ara-CTP, which act as alternative toxic nucleotides that are incorporated into the forming DNA from active transcription (Cros *et al.*, 2004). Ara-C disrupts cell replication by chain termination from the inability to bind further nucleotides for polymerase extension, along with DNA polymerase inhibition. Sustained replication stalling results in disintegration of the replication complex, and DNA repair mechanisms exist to ensure fidelity of the unwound DNA within the transcription bubble. During DSB repair, Rad50 and the MRN complex, which comprises of a protein trimer of MRE11-RAD50-NBS1, accumulate in response to DNA double-stranded breaks in the chromosome (Yuan and Chen, 2010). This complex is able to bind to damaged chromatin via the phosphorylation of MDC1 (Spycher *et al.*, 2008). Interestingly, Rad50 was identified as a potential dsDNA-binding target in the mass spectrometry analysis of cytosolic lysate immunoprecipitation using dsDNA antibody. Rad50 activity is dependent on ATP (Kinoshita *et al.*, 2009) and the MRN repair complex translocates to the cytoplasm following heat-shock and irradiation (Seno and Dynlacht, 2004). Thus cell stress induced by Ara-C along with dsDNA-bound proteins identified to be involved in early DSB repair suggest a role of early DNA repair in the accumulation of cytosolic dsDNA.

Of note, Ara-C was dissolved in DMSO, which is known to affect cell membrane permeability (David, 1972; Shen *et al.*, 2006). DMSO treatment was used as a negative control of Ara-C treatment, by adding the same volume of drug or

control. For example, 10uM (0.1% v/v.) of DMSO was added as control for Ara-C treatment. However, experiments with fixation of cells for cytosolic DNA, as well as for RNase H treatment on RNA:DNA hybrids, were performed in the absence of DMSO. These showed clear RNA:DNA hybrid staining, suggesting the reliability of the antibody stain. Further, comparison between Ara-C and DMSO treated cells clearly showed an increase in cytosolic staining, suggesting that Ara-C indeed resulted in increase of cytosolic DNA in cells after treatment, independent of any effect by DMSO.

Another way by which cytosolic dsDNA could occur is with reverse transcription of transposons. LINE-1 retrotransposons were able to induce DSB in excess of LINE-1 insertions back into the genome (Gasior *et al.*, 2006) in response to DNA damage, providing a route by which dsDNA and related nucleic acids could accumulate within the cell. As reverse transcription may occur independently of DNA damage, this may provide another explanation for the low levels of cytosolic dsDNA seen before addition of DNA damage agents.

7.3 Detection of nucleic acids by staining

To examine nucleic acid stains in the cells, a variety of nucleic acid stains was utilized. To distinguish between dsDNA and RNA:DNA hybrids, specific antibodies were primarily used. The PicoGreen dye used could have less distinction between the structure difference of dsDNA and RNA:DNA, which may affect the specificity of its intercalating nature.

dsDNA antibodies were specific against cytosolic dsDNA, with either a weak or non-existent nuclear stain. We speculate that the cytosolic dsDNA that is recognized by the dsDNA antibody has been processed and is structurally or epigenetically different from the nuclear DNA from which it is derived. This would explain the weaker nuclear stain and distinct dsDNA cytosolic stain we see when using the dsDNA antibody for staining. dsDNA antibodies are essential in the

diagnosis of certain autoimmune diseases, such as systemic lupus erythematosus syndrome. The dsDNA antibody clone, AE-2, was used to stain dsDNA of viral Porcine circovirus type 2 (PCV2) infecting lymph nodes and thymus of gnotobiotic piglets. In PCV-2 infected sections, cells in thymi were detected for cytoplasmic staining in addition to nuclei stain, and staining was specific to infected cells, indicating that the antibody might also have specificity for non-nuclear dsDNA (Hamberg *et al.*, 2007).

RNA:DNA hybrid antibodies (S9.6) have been widely used in publications in the detection of hybrid structures in the cell (Boguslawski *et al.*, 1986; Hu *et al.*, 2006; Phillips *et al.*, 2013). The kinetics and binding ability of the antibody has also been determined (Phillips *et al.*, 2013), and it has been particularly useful in the area of detecting small RNA after probing on microarrays, down to 15 nucleotide length (Hu *et al.*, 2006). This enables it to detect short strand RNA:DNA hybrids that might have escaped the detection by unspecific binding of dsDNA antibodies. This could also be one reason why the S9.6 cytosolic staining is more intense than dsDNA staining. Further, S9.6 antibody was also shown to have higher affinity for poly(I):poly(dC) hybrids as compared to poly(A):poly(dT) hybrids, suggesting that it might contain sequence specificity for its recognition criteria (Hu *et al.*, 2006). While cytosolic RNA:DNA hybrids in human cells were described as viral or bacterial-derived in previous published studies (Kailasan Vanaja *et al.*, 2014; Rigby *et al.*, 2014), a study also suggested the presence of extranuclear RNA:DNA hybrids in yeast (Nakama *et al.*, 2012). The authors speculated that the RNA:DNA hybrids are located in mitochondria as RNA:DNA hybrids were reported to form throughout the mitochondrial genome (Brown *et al.*, 2008). However, staining of A549 cells for RNA:DNA hybrids and, mitochondrial markers showed that RNA:DNA hybrids are localized outside of mitochondria. RNA:DNA hybrids are also transiently formed during transcription and replication of genomic DNA (Kireeva *et al.*, 2000; Aguilera and Garcia-Muse, 2012), however these are POL II-driven and located in the nucleus.

We also found that cytosolic RNA:DNA hybrids in tumor cells depend on transcription by POL III, while ssDNA and dsDNA are generated by DNA damage response-initiated events. However, it is conceivable that RNA:DNA hybrid contribute to the presence of ssDNA and dsDNA in tumor cells by stalling replication forks, which results in DNA damage and genomic instability (Hamperl and Cimprich, 2014). Consistent with this conclusion, DNA damage response is activated in *Rnaseh2*-deficient cells (Hiller *et al.*, 2012). Furthermore, overexpression of *Rnaseh1*, which degrades the RNA strand in RNA:DNA hybrids, decreased the levels of cytosolic ssDNA and dsDNA in tumor cells (Shen et al., manuscript submitted).

PicoGreen has a chemical structure that is able to intercalate with dsDNA in sensitive amounts. PicoGreen fluorescence increases more than 1000-fold after binding to DNA; and PicoGreen dye exhibits high sensitivity, recognizing both polymeric DNA and short duplexes less than 20 base pairs. DNase I treatment decreased PicoGreen stain on nucleotides (Choi and Szoka, 2000). PicoGreen forms electrostatic contacts with DNA phosphate group, binding to the minor groove of DNA. PicoGreen spans four base pairs of the DNA structure, and its benzo-thiazol group has electrostatic interactions with DNA phosphate groups (Dragan et al., 2010). This suggests that PicoGreen binding recognition is based upon the DNA phosphate backbone, and has enriched binding to nucleotides. PicoGreen reagent also shows no bias towards either GC or AT rich regions, with both homopolymers having similar quantum yields of fluorescence efficiency when bound to PicoGreen (Singer *et al.*, 1997). However, experimental results of cells treated with RNase H showed a decrease in PicoGreen signal in the cytosol (Fig. 4.1), suggesting that PicoGreen might also recognize RNA:DNA hybrids, given the specificity of RNase H (Keller and Crouch, 1972). This is also supported by previous reports that showed PicoGreen also stains dsRNA at ten-times lower intensity than dsDNA (Singer *et al.*, 1997), suggesting that RNA:DNA hybrids might also be recognized by the dye. Thus PicoGreen might not exclusively recognize dsDNA, and is able to stain for

RNA:DNA hybrids in cells. While strong nuclear signal of PicoGreen suggested that the large amounts of nuclear genomic dsDNA were stained, PicoGreen had high sensitivity that also allowed it to stain for less intense cytosolic signals in addition to the nuclear genome.

In contrast, DAPI is an A-T sensitive dye that intercalates with the minor groove of DNA to form a fluorescent complex. Similarly, Hoechst dye 33258 interacts preferentially with the minor groove of AT-rich sequences of a DNA duplex (Haq *et al.*, 1997); showing more fluorescent yield as compared to DAPI. These two nuclear dyes are more commonly used for microscopy staining, however their sensitivity is less as compared to PicoGreen, partly due to their selective specificity. Furthermore, it is known that high nuclear staining of Hoechst can obscure mitochondria staining (Ligasova *et al.*, 2013). Similarly, stains of cytosolic DNA would appear much weaker, and hence can be masked by strong Hoechst nuclear signal, a possible explanation for why DAPI and Hoechst could not stain for cytosolic DNA.

7.4 Nature of dsDNA interactions with cytoplasmic proteins

To examine the retention of DNA in the cytosol, a selection of DNA-binding proteins, DNA sensors, and compartmental proteins were co-stained with the DNA. However a representative colocalization of dsDNA antibody with another protein could not be identified. dsDNA is known to be stable in solution, and increases upon chaperoning with a binding protein (Williams *et al.*, 2002). In addition, the presence of DNA in the cytosol seems contradictory to the body's adverse reaction to aberrant endogenous DNA that is involved in inflammatory conditions.

Different forms of DNase are found throughout the cell to ensure digestion of endogenous DNA (Hornung and Latz, 2010). DNase I is secreted into the extracellular space to prevent accumulation of DNA-containing immune complexes, and DNase II is found in acidic lysosomes that engulf apoptotic or necrotic cells.

DNase III (TREX1) is the only cytosolic DNase that is located on the endoplasmic reticulum. However, the presence of cytosolic DNA in cancer cell lines may suggest that DNA damage might cause a high turnover of DNA such that degradation is unable to keep up with its accumulation. In cells with particularly high rates of replication, fork stalling and translation may result in more DNA damage.

Another possibility for cytosolic DNA accumulation in cancer cells lines, is the presence of defective TREX I that prevents DNA degradation. It is known the *Trex1* knockout mice exhibit inflammation and have lower rates of survival (Morita *et al.*, 2004), while TREX1 is associated in human autoimmune disease (Kavanagh *et al.*, 2008). However, TREX1 is a 3' to 5' exonuclease that degrades ssDNA (Yang *et al.*, 2007), although it is also said to act in concert with NM23-H1 endonuclease at nicked DNA regions (Chowdhury *et al.*, 2006). The ssDNA is primarily produced through G1/S transition, as shown by the constitutive ATM-dependent checkpoint activation in deficient cells, and accumulates at the replication fork for TREX 1 to process. Hence if the cytosolic DNA is dsDNA, the TREX1 might not be able to recognize it as a suitable substrate for degradation, and its activity should not affect the stability of replication sites and DNA resection, which occurs during DSB repair.

Another possibility is the presence of DNA-binding proteins that stabilize the cytosolic dsDNA and prevent its recognition by endonucleases. This is critical if the cytosolic DNA is functional in the cell. As DNA is able to encode information within a short sequence, it is perhaps a possibility that presence of DNA in damaged cells might serve a functional purpose.

An immediate candidate for cytosolic DNA-binding proteins is the family of histones. Given that several lines of evidence show cytosolic DNA originating from the nucleus, it would be logical for histones to bind the DNA as it is chaperoned into the cytosol. In particular, citrullinated H3 was hypothesized to be a marker for cytosolic DNA as it was shown to be associated with transcription interference, and

thus sites of DNA damage. However, no colocalization was observed, even for H3 citrullinated proteins.

7.5 RNA POL III-dependent cytosolic RNA:DNA hybrids

Each POL in the RNA POL family of proteins has defined transcriptional roles, where RNA POL I transcribes ribosomal RNA, RNA POL II transcribes protein-encoding mRNA, while RNA POL III transcribes 5S rRNA, tRNA, and certain retroelements (Weinmann and Roeder, 1974; Sentenac, 1985; Shilatifard, 1998; Kuhn *et al.*, 2007). Eukaryotic non-coding RNAs can be transcribed by all three polymerases (Goodrich and Kugel, 2006). Interestingly, inhibition of RNA POL III, abrogated the presence of cytosolic RNA:DNA hybrids suggesting that RNA POL III transcripts are required for the existence of such structures in the cytosol. In particular, nuclear RNA:DNA hybrids were not decreased after this treatment, consistent with previous reports that RNA:DNA hybrids in the nucleus are mainly from R-loops, a structure that is highly associated only with RNA POL II transcription machinery (Skourti-Stathaki *et al.*, 2011). This raises the specificity of cytosolic RNA:DNA hybrids as RNA POL III-driven as opposed to nuclear RNA:DNA hybrids being mainly RNA POL II-driven.

RNA POL III is shown to increase SINE transcription upon heat shock, a form of cell stress, and these transcripts can repress mRNA transcription (Ponicsan *et al.*, 2010). This presents a form of gene regulation via POL III; in particular RNA:DNA hybrids are most likely formed through reverse-transcription of retrotransposons Recently, endogenous siRNAs (endo-siRNA) were shown to be present in cells, and these endo-siRNAs were decreased in human breast cancer cells as compared to normal breast cells (Chen *et al.*, 2012), along with expression of endo-siRNAs being able to silence LINE-1 retrotransposons through increased DNA methylation. RNA polymerase III transcribes non-coding RNAs that may be

processed into endo-siRNAs. In this way, RNA:DNA hybrids that bind to AGO2 may be a product of this inhibition, as endo-siRNAs are derived from mobile elements in the genome. However, previous research within the lab performed overexpression of open reading frame (ORF) 1 and ORF2 proteins in cells, where these proteins are encoded by LINE-1 and function to help retrotransposition (Feng *et al.*, 1996; Moran *et al.*, 1996). These cells showed no change in cytosolic DNA levels, suggesting that reverse transcription of retroelements did not play a role in the accumulation of these cytosolic nucleic acids.

Although MRC-5 cells were shown to have cytosolic DNA, they were invariant to RNA POL III expression. BRCA1 has been shown to inhibit RNA POL III transcription in a cell specific manner (also in Hela) (Veras *et al.*, 2009), hence RNA POL III-driving of RNA:DNA hybrids might be cell type specific. We have shown that endogenous RNA:DNA hybrids are present in human lymphoma tissues. Presence of EBV infection in the patient could be a possible explanation for high amounts of RNA:DNA hybrids, however the datasheet of the tissues that showed the exact stage and type of lymphoma, did not state that the tissues were EBV infected. Further, we have also stained other tissue samples and found that pancreatic cancer tissues exhibit similar specificity of RNA:DNA hybrid accumulation. Nevertheless, the role of RNA POL III in transcription and overexpression leads to high proliferation and tumorigenesis, relating its relevance to tumorigenesis.

7.6 Role of DDX17 in DNA damage and miRNA biogenesis

DDX17 has been shown to be involved in transcriptional regulation in addition to its role in miRNA processing. DDX17 along with its related DDX5 protein have been associated with cancer, as they modulate p53, and Drosha, to regulate processing of oncomirs (Bates *et al.*, 2005; Dardenne *et al.*, 2014). DDX17 has also previously been implicated in cell proliferation towards tumorigenesis (Dardenne *et al.*, 2012). In addition, DDX5 also activates p53 tumor suppressor in

response to DNA damage, but is tissue and context-dependent for the induction of apoptosis (Nicol *et al.*, 2012). I detected the presence of peptides from putative DDX5/DDX17 in both dsDNA and RNA:DNA hybrid immunoprecipitated cytosolic lysates, in mass spectrometry. Further, *DDX17* knockdown increased cytosolic dsDNA stains in cells, and it was shown via immunoblot that DDX17 protein bound to RNA:DNA hybrids. Off-target effects might have been an explanation for increase of cytosolic DNA after siRNA. However, we used NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to analyse the siDDX17 #3 sequence, which showed 100% sequence coverage towards *DDX17*, with the next sequence-compatible protein (KRAB-A domain containing 2, KRBA2) matching only 66% query coverage. On the other hand, siDDX17 #1 sequence had the next sequence-compatible protein (heterochromatin protein 1, binding protein 3, HP1BP3) matching 71% query coverage. This suggested that siDDX17 #3 had less off-target effects based on its sequence specificity. Interestingly, previous studies showed that *DDX17* knockout mouse embryo fibroblasts also had reduced ribosomal RNA (rRNA) expression (Fukuda *et al.*, 2007), similar to the disappearance of cytosolic dsDNA in response to *DDX17* knockdown in A549 cells. One possible explanation could be the accumulation of dsDNA and RNA:DNA hybrids is dependent on *DDX17* expression.

Recently, DDX17 has been shown to be a multi-tasking regulator (Fuller-Pace, 2013), and DDX5/DDX17 helicase activity was hypothesized to favor binding to G-quadruplexes (Dardenne *et al.*, 2014). Nevertheless, although DDX17 is a putative RNA helicase, it is not known if this function extends for DNA. Further, DDX17 also regulates gene expression by regulating alternative splicing of DNA-binding factors such as macroH2A1 (Dardenne *et al.*, 2012), suggesting another role of DDX17 that might contribute to the release of cytosolic nucleic acids. We showed that DNA damage associated with upregulation of cytosolic DNA and DDX17, yet DDX17 knockdown resulted in increased cytosolic dsDNA. This conundrum has yet to be fully addressed; however we suggest an explanation as follows. DDX17 is a

RNA helicase, and it is also known to play a variety of roles in relation to tumorigenesis, as reviewed by Fuller-Pace and Moore (Fuller-Pace and Moore, 2011). It could be possible that the function of DDX17 may differ according to the location in which it is found. DDX17 is a RNA helicase that is able to separate dsRNA to single strands (Lin *et al.*, 2005), and it may also help to unwind dsDNA in the cytosol, thus degrading cytosolic dsDNA. However in the presence of Ara-C damage, DDX17 may be unable to cope with the increase of cytosolic dsDNA due to widespread nuclear DNA damage. Further experiments such as inhibition of nuclear export in the presence of DNA damage would address such questions.

Moreover, as dsDNA and RNA:DNA hybrids were shown to be dependent on different mechanisms (DDR-dependent vs. non-DDR-dependent), one might postulate that DDX17 might affect the accumulation of these two species of nucleic acids in different mechanisms. This might be possible due to the multi-functional roles of DDX17.

7.7 MiRNA expression and RNA:DNA hybrids

MiRNAs require export from the nucleus to the cytoplasm as part of their biogenesis. DNA damage induces an ATM-dependent miRNA expression profile that is regulated by transport through the exportin-5 (XPO-5) bound nuclear pore complex (Wan *et al.*, 2013). While DNA damage increased dsDNA cytosolic accumulation, RNA:DNA hybrids were not found to be highly accumulated in the cytosol in response. This suggested separate pathways of regulation for these two types of nucleic acids in response to DNA damage.

RNA POL III was previously found to affect miRNA accumulation. Here, we show that inhibition of RNA POL III activity affected miRNA expression, with affected gene targets regulated in pathways in RNA transport and RNA surveillance. Analysis of the miRNA expression profile showed that RNA POL III modulates the

expression of a small number of miRNAs, most of which were upregulated upon RNA POL III inhibition, suggesting a complex regulation of these miRNAs by RNA POL III in A549 cells. Surprisingly, RNA POL III inhibition reduced the expression of only four miRNAs. Three out of the four miRNAs were also downregulated by Ara-C, which does not modulate the levels of cytosolic RNA:DNA hybrids. Hence, our data would suggest that cytosolic RNA:DNA hybrids consist of very limited number of POL III-transcribed miRNAs, possibly only miR-4499.

Alternatively, it is conceivable that POL III-modulated miRNAs target transcripts of genes, which regulate the presence of cytosolic RNA:DNA hybrids. Consistent with this possibility, many POL III modulated miRNAs potentially target RNA transport and mRNA stability pathways. Cytosolic RNA:DNA hybrids may also be generated by the transcription of cytosolic DNA by POL III, which was reported to detect cytosolic DNA and induce type I interferons through the RIG-I pathway (Schwartz *et al.*, 1974; Jaehning and Roeder, 1977; Chiu *et al.*, 2009). However, POL III did not co-localize with cytosolic DNA or cytosolic RNA:DNA hybrids.

MiRNA has been associated with tumorigenesis and cancer. Further, dysregulated RNA POL III correlates with tumor progression, and RNA POL III transcribed open reading frames occur with cancerous cell lines, suggesting an increase in transcription of genes in response to RNA POL III, thus providing a mechanism to increase generation of RNA:DNA hybrids.

A pathway analysis of potential target genes from the identified miRNAs showed that RNA transport and mRNA surveillance pathways were affected. This is consistent with the possibility that regulation of miRNAs and RNA:DNA hybrids via RNA POL III affects cancer progression. Some genes of these pathways were further quantified to respond to RNA POL III treatment in accordance with the miRNA microarray analysis.

Finally, it is conceivable that reverse transcription of RNA POL III-transcribed retroelements produces RNA:DNA intermediates in the cytosol similar to replicating RNA viruses. It would be interesting to investigate mir-4499 transcription or expression, which is more specific to RNA POL III regulation. A functional knockdown of the mir-4499 to determine its effect on cytosolic RNA:DNA hybrids might help to uncover its role in response to RNA POL III inhibition.

7.8 Innate immune signaling of nucleic acids

Nucleic acids are key activators of the innate immune system, with intracellular DNA and RNA acting as PAMPs during infection, and DAMPs after DNA damage. Here, we found the presence of dsDNA and RNA:DNA hybrids in tumorigenic cell lines. The significance of these cytosolic nucleic acids has yet to hint at a role for innate signaling. Although interferon levels were not significantly affected after treatment of RNA POL III that inhibits RNA:DNA hybrid accumulation, little is known if differential accumulation of RNA:DNA hybrids may affect tumor recognition in vivo. Alternatively, other pathways of immune signaling may also play a role in RNA:DNA hybrid sensing; the NLRP3 inflammasome pathway was shown to be activated by bacterial RNA:DNA hybrids inducing IL-1β production (Kailasan Vanaja *et al.*, 2014). Intriguingly, we also observed a distinct detection of RNA:DNA hybrids in cancer tissues as compared to normal tissues, suggesting that RNA:DNA hybrids were selectively present in a cancer microenvironment. Further, as RNA:DNA hybrids were shown to activate the pathways involved in immune sensing (Kailasan Vanaja *et al.*, 2014; Rigby *et al.*, 2014), it is postulated that the presence of RNA:DNA hybrids in cancer tissues might activate a similar immune response. Given the correlation of the immune system with cancer, as reviewed by Schulz (Schulz, 2009), it would be interesting to examine the recruitment of immune cells in response to the presence of RNA:DNA hybrids at cancer sites or sites of DNA damage *in vivo*.

In addition to intracellular DNA, release of DNA from dying cells via necrosis is also recognized as a 'danger' signal in response to treatment of alum as a vaccine adjuvant (Marichal *et al.*, 2011). In autoimmune diseases such as SLE, dysregulation of DNases play a major role in disease progression. While we tried to express DNase I in the cytosol by tagging the protein to the cell membrane, cells expressing DNase I exhibited high levels of cell death, possibly due to degradation of the genome during replication, where the nuclear envelope disintegrates and the genomic DNA is exposed to highly expressed cytosolic DNase I. This suggests that mechanism of cytosolic DNA production proceeds at a rapid rate as compared to degradation by DNase I. Another possibility is the presence of cytosolic DNA that is stably expressed with modifications that prevent it from being degraded by DNase I.

DNase II is predominantly expressed in lysosomes and exhibits high kinetics at low pH (Liu *et al.*, 2008). It functions to degrade apoptotic DNA engulfed from nearby 'dying' cells, and its importance is underscored by the fact that DNase II-deficient mice are embryonically lethal, with undigested DNA accumulated in macrophages along with high levels of interferon-beta (Okabe *et al.*, 2005; Yoshida *et al.*, 2005). However staining for lysosomes and other compartment markers along with dsDNA found no distinct correlation between the two stains, suggesting that dsDNA did not accumulate in these compartments, thus DNase II might not have a regulatory role in the production of dsDNA or these cytosolic nucleic acids.

Previous studies have shown the presence of DNA damage along with cytosolic DNA in murine tumor cells, suggesting that cytosolic DNA functions as an endogenous DAMP trigger for the immune response to affect tumor progression (Lam *et al.*, 2014a). In my study, I focused on the presence of cytosolic nucleic acids in human cancer cell lines. Interestingly, the THP-1 human monocytic cell line derived from acute monocytic leukemia also stained positive for RNA:DNA hybrids. As such, tracking of tumor progression in the B-cell lymphoma mouse model, Eμ-*Myc* mice, with the presence of RNA POL III and its effect on cytosolic RNA:DNA

accumulation might provide insight into how levels of RNA:DNA associate with the initial regression of Eμ-*Myc* B cell tumors and eventual reemergence of the leukemia. In addition, while RNA:DNA has been shown to be immunologically active in DCs (Rigby *et al.*, 2014), its role in fibroblasts and other immune cells have yet to be analyzed. An understanding of the effect of RNA:DNA hybrid accumulation in modulating immune response would open up an alternative substrate to DNA sensors in the detection of DAMPs in tumorigenic cells.

7.9 Tumor progression in Eμ-*Myc* mice

Translational suppression occurs with integrated stress upon cells and the formation of stress granules in the cytoplasm that contain high amounts of RNA from stalled ribosomes (Buchan and Parker, 2009). ZBP1 was found to colocalize with stress granules in response to oxidative stress, and although it was not required for stress granule assembly (Stohr et al., 2006). It was found to associate and stabilize mRNA transcripts in stress granules. ZBP1 intracellular localization was dependent on its Z-DNA binding domain, where mutation of that domain resulted in a different cytosolic distribution of ZBP1 in cells, in response to stress (Deigendesch *et al.*, 2006). ZBP1 was also found to be a cytosolic DNA sensor signaling through the TBK1/IRF3 pathway to activate IFN (Takaoka *et al.*, 2007). Interestingly, data from our lab showed that Zbp1-/-Eμ-Myc mice displayed lower amounts of cytosolic dsDNA in tumor cells, in contrast to Eμ-Myc mice (Lam et al., manuscript submitted). Thus, in order to investigate if the phenotype of B cell lymphoma in Eμ-Myc mice is due to the ZBP1 signaling pathway through TBK1 with IRF3, I crossed

Tnfa/Tbk1, and *Tnfa*-deficient mice, with Eμ-*Myc* mice. In addition to ZBP1, TBK1 is also a mediator of other DNA sensing pathways, as it was able to mount an interferon response to DNA in the absence of ZBP1 (Takaoka *et al.*, 2007; Ishii *et al.*, 2008) TBK1 was activated in response to DNA damage, and this process was dependent on the DDR (Lam *et al.*, 2014a). TNF is a proinflammatory cytokine that

is activated in response to DNA sensing (Ivanov *et al.*, 2007), and stimulate immune cells such as neutrophils and macrophages (Peterson *et al.*, 2006). Hence, we also determined if *Tnfa* deficiency could affect tumor progression Eμ-Myc mice.

Results showed that *Tnfa/Tbk1* and *Tnfa*-deficient Eμ-*Myc* mice exhibited higher and rapid mortality rates with a high tumor load even during regression phase from day 40-60. TNF itself is able to activate pathways leading to induction of proinflammatory cytokines or genes involved with survival (Balkwill, 2009). Recombinant TNF is able to cause necrosis of tumors, along with the activation of cell killing via CD8+ T cell and NK cells (Kashii *et al.*, 1999; Prevost-Blondel *et al.*, 2000). Given that Eμ-*Myc* mice are dependent upon NK and T cells for tumor cell killing (Croxford *et al.*, 2013), decreased *Tnfa* expression affected tumor load in Eμ-*Myc* mice, consistent with observations. The effect of TNF may mask the contribution of TBK1 towards progression of B cell tumors in mice, however the surprisingly low tumor load of *Tnfa*$^{-/-}$*Tbk1*$^{-/-}$Eμ-*Myc* mice suggests other regulatory mechanisms that replace this pathway in the absence of both TNF and TBK1.

In this thesis, we showed that siRNA of RNA Pol III, and Pol III inhibitor treatment did not reduce interferon mRNA expression, suggesting that the interferon pathway was not activated via cytosolic RNA:DNA hybrids. TNF and interferon levels triggered by poly (dA:dT) were previously found to be dependent on RIG-I and RNA polymerase III, a purported DNA sensor (Ablasser *et al.*, 2009). However, IFNα release in infected plasmacytoid DCs (pDCs) was also found to be suppressed by TNF (Palucka *et al.*, 2005). A possilibity for our lack of interferon detection could be due to RNA:DNA hybrids activating TNF secretion instead of IFNα in cancer cells. This would suggest that TNF might act as another arm of immunosurveillance in Eμ-*Myc* mouse tumors.

Recently, cGAS was identified to be the second messenger that transduces the sensing cytosolic DNA into interferon expression for host immune response (Gao

et al., 2013; Li *et al.*, 2013; Sun *et al.*, 2013). Further, STING as an adaptor protein in phosphorylation of IRF3 was confirmed to act downstream of cGAS activation, providing a new perspective of DNA sensing (Wu *et al.*, 2013). The existence of this pathway elevates the importance of STING as a linchpin protein in the activation of the interferon response. Thus it would be interesting to cross Eμ-*Myc* mice with *Sting*-deficient mice and observe tumor regression phase. Although we did not observe strong colocalization of STING and cytosolic dsDNA, STING might act downstream of a DNA sensor of dsDNA to trigger an immune response. Thus, one might expect that loss of STING leads to higher accumulation of cytosolic DNA without activation of the host immune system, allowing for rapid proliferation of B cell tumors without any regression phase as NK and T cells are unable to recognize signals for tumor-cell mediating killing. Given that the *Tnfa*-deficient Eμ-*Myc* mice died earlier, the activation of the immune response serves as an important player in the mortality of B cell tumors, and STING might represent a possible key player in tumorigenesis. Tumor progression of *Sting*-deficient Eμ-*Myc* would therefore present an interesting model to study the pathway of tumor sensing.

7.10 Conclusion

In summary, our results demonstrate the presence of cytosolic dsDNA and the first instance of RNA:DNA hybrids in a variety non-infected human cells, and in human lymphoma tissues for RNA:DNA hybrids. While cytosolic dsDNA is dependent upon DDR, RNA:DNA hybrids are independent of DDR, but are instead regulated by RNA POL III. Further, RNA:DNA hybrids bind to proteins of the miRNA machinery, DDX17 and AGO2. POL III inhibition also targets miRNAs involved in RNA transport and surveillance. Investigation of Eμ-*Myc* mice tumorigenesis revealed the importance of TNF-α in tumor cell killing, while the function of TBK1 remains to be revealed. These findings extend the knowledge in the field of nucleic acids in cells for DNA-sensing, and enhance the characterization of molecules that can be targeted for cancer immunotherapy.

Bibliography

Abbink, T.E., and Berkhout, B. (2008). HIV-1 reverse transcription initiation: a potential target for novel antivirals? Virus Res *134*, 4-18.

Ablasser, A., Bauernfeind, F., Hartmann, G., Latz, E., Fitzgerald, K.A., and Hornung, V. (2009). RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. Nat Immunol *10*, 1065-1072.

Adachi, S., Obaya, A.J., Han, Z., Ramos-Desimone, N., Wyche, J.H., and Sedivy, J.M. (2001). c-Myc is necessary for DNA damage-induced apoptosis in the G(2) phase of the cell cycle. Mol Cell Biol *21*, 4929-4937.

Adams, J.M., Harris, A.W., Pinkert, C.A., Corcoran, L.M., Alexander, W.S., Cory, S., Palmiter, R.D., and Brinster, R.L. (1985). The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature *318*, 533-538.

Aguilera, A., and Garcia-Muse, T. (2012). R loops: from transcription byproducts to threats to genome stability. Mol Cell *46*, 115-124.

Aguilera, A., and Gomez-Gonzalez, B. (2008). Genome instability: a mechanistic view of its causes and consequences. Nat Rev Genet *9*, 204-217.

Amiot, F., Boussadia, O., Cases, S., Fitting, C., Lebastard, M., Cavaillon, J.M., Milon, G., and Dautry, F. (1997). Mice heterozygous for a deletion of the tumor necrosis factor-alpha and lymphotoxin-alpha genes: biological importance of a nonlinear response of tumor necrosis factor-alpha to gene dosage. Eur J Immunol *27*, 1035-1042.

Anand, P.K. (2010). Exosomal membrane molecules are potent immune response modulators. Commun Integr Biol *3*, 405-408.

Anderson, M.S., and Bluestone, J.A. (2005). The NOD mouse: a model of immune dysregulation. Annu Rev Immunol *23*, 447-485.

Ashley, N., Harris, D., and Poulton, J. (2005). Detection of mitochondrial DNA depletion in living human cells using PicoGreen staining. Exp Cell Res *303*, 432-446.

Babashah, S., and Soleimani, M. (2011). The oncogenic and tumour suppressive roles of microRNAs in cancer and apoptosis. Eur J Cancer *47*, 1127-1137.

Bacolla, A., Wojciechowska, M., Kosmider, B., Larson, J.E., and Wells, R.D. (2006). The involvement of non-B DNA structures in gross chromosomal rearrangements. DNA Repair (Amst) *5*, 1161-1170.

Balkwill, F. (2009). Tumour necrosis factor and cancer. Nat Rev Cancer *9*, 361-371.

Banerjea, A., Ahmed, S., Hands, R.E., Huang, F., Han, X., Shaw, P.M., Feakins, R., Bustin, S.A., and Dorudi, S. (2004). Colorectal cancers with microsatellite instability display mRNA expression signatures characteristic of increased immunogenicity. Mol Cancer *3*, 21.

Barber, G.N. (2014). STING-dependent cytosolic DNA sensing pathways. Trends Immunol *35*, 88-93.

Bates, G.J., Nicol, S.M., Wilson, B.J., Jacobs, A.M., Bourdon, J.C., Wardrop, J., Gregory, D.J., Lane, D.P., Perkins, N.D., and Fuller-Pace, F.V. (2005). The DEAD box protein p68: a novel transcriptional coactivator of the p53 tumour suppressor. Embo J *24*, 543-553.

Beletskii, A., and Bhagwat, A.S. (1996). Transcription-induced mutations: increase in C to T mutations in the nontranscribed strand during transcription in Escherichia coli. Proc Natl Acad Sci U S A *93*, 13919-13924.

Belgnaoui, S.M., Gosden, R.G., Semmes, O.J., and Haoudi, A. (2006). Human LINE-1 retrotransposon induces DNA damage and apoptosis in cancer cells. Cancer Cell Int *6*, 13.

Bianchi, M.E. (2007). DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol *81*, 1-5.

Biffi, G., Tannahill, D., McCafferty, J., and Balasubramanian, S. (2013). Quantitative visualization of DNA G-quadruplex structures in human cells. Nat Chem *5*, 182-186.

Biton, S., and Ashkenazi, A. (2011). NEMO and RIP1 control cell fate in response to extensive DNA damage via TNF-alpha feedforward signaling. Cell *145*, 92-103.

Boguslawski, S.J., Smith, D.E., Michalak, M.A., Mickelson, K.E., Yehle, C.O., Patterson, W.L., and Carrico, R.J. (1986). Characterization of monoclonal antibody to DNA.RNA and its application to immunodetection of hybrids. J Immunol Methods *89*, 123-130.

Bolstad, B.M., Irizarry, R.A., Astrand, M., and Speed, T.P. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics *19*, 185-193.

Borchert, G.M., Lanier, W., and Davidson, B.L. (2006). RNA polymerase III transcribes human microRNAs. Nat Struct Mol Biol *13*, 1097-1101.

Boyd, J.H., Kan, B., Roberts, H., Wang, Y., and Walley, K.R. (2008). S100A8 and S100A9 mediate endotoxin-induced cardiomyocyte dysfunction via the receptor for advanced glycation end products. Circ Res *102*, 1239-1246.

Braun, C.J., Zhang, X., Savelyeva, I., Wolff, S., Moll, U.M., Schepeler, T., Orntoft, T.F., Andersen, C.L., and Dobbelstein, M. (2008). p53-Responsive micrornas 192 and 215 are capable of inducing cell cycle arrest. Cancer Res *68*, 10094-10104.

Bretz, N.P., Ridinger, J., Rupp, A.K., Rimbach, K., Keller, S., Rupp, C., Marme, F., Umansky, L., Umansky, V., Eigenbrod, T., Sammar, M., and Altevogt, P. (2013). Body fluid exosomes promote secretion of inflammatory cytokines in monocytic cells via Toll-like receptor signaling. J Biol Chem *288*, 36691-36702.

Brown, T.A., Tkachuk, A.N., and Clayton, D.A. (2008). Native R-loops persist throughout the mouse mitochondrial DNA genome. The Journal of biological chemistry *283*, 36743-36751.

Buchan, J.R., and Parker, R. (2009). Eukaryotic stress granules: the ins and outs of translation. Mol Cell *36*, 932-941.

Burge, S., Parkinson, G.N., Hazel, P., Todd, A.K., and Neidle, S. (2006). Quadruplex DNA: sequence, topology and structure. Nucleic Acids Res *34*, 5402-5415.

Burgers, P.M. (2009). Polymerase dynamics at the eukaryotic DNA replication fork. J Biol Chem *284*, 4041-4045.

Burrow, A.A., Marullo, A., Holder, L.R., and Wang, Y.H. (2010). Secondary structure formation and DNA instability at fragile site FRA16B. Nucleic Acids Res *38*, 2865-2877.

Byers, S.A., Schafer, B., Sappal, D.S., Brown, J., and Price, D.H. (2005). The antiproliferative agent MLN944 preferentially inhibits transcription. Mol Cancer Ther *4*, 1260-1267.

Bygrave, A.E., Rose, K.L., Cortes-Hernandez, J., Warren, J., Rigby, R.J., Cook, H.T., Walport, M.J., Vyse, T.J., and Botto, M. (2004). Spontaneous autoimmunity in 129 and C57BL/6 mice-implications for autoimmunity described in gene-targeted mice. PLoS Biol *2*, E243.

Caldecott, K.W. (2008). Single-strand break repair and genetic disease. Nat Rev Genet *9*, 619-631.

Calin, G.A., Sevignani, C., Dumitru, C.D., Hyslop, T., Noch, E., Yendamuri, S., Shimizu, M., Rattan, S., Bullrich, F., Negrini, M., and Croce, C.M. (2004). Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci U S A *101*, 2999-3004.

Cancer, I.A.f.R.o. (2014). GLOBOCAN 2012: estimated cancer incidence, mortality and prevalence worldwide in 2012. World Health Organization. http://globocan. iarc. fr/Pages/fact_sheets_cancer. aspx. Accessed on *9*.

Canella, D., Praz, V., Reina, J.H., Cousin, P., and Hernandez, N. (2010). Defining the RNA polymerase III transcriptome: Genome-wide localization of the RNA polymerase III transcription machinery in human cells. Genome Res *20*, 710-721.

Carey, M.F., Singh, K., Botchan, M., and Cozzarelli, N.R. (1986). Induction of specific transcription by RNA polymerase III in transformed cells. Mol Cell Biol *6*, 3068-3076.

Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N., and Williamson, B. (1975). An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci U S A *72*, 3666-3670.

Cerritelli, S.M., Frolova, E.G., Feng, C., Grinberg, A., Love, P.E., and Crouch, R.J. (2003). Failure to produce mitochondrial DNA results in embryonic lethality in Rnaseh1 null mice. Mol Cell *11*, 807-815.

Chan, Y.A., Hieter, P., and Stirling, P.C. (2014). Mechanisms of genome instability induced by RNA-processing defects. Trends Genet.

Chatterjee, S., and Grosshans, H. (2009). Active turnover modulates mature microRNA activity in Caenorhabditis elegans. Nature *461*, 546-549.

Chauhan, S.K., Singh, V.V., Rai, R., Rai, M., and Rai, G. (2014). Differential microRNA profile and post-transcriptional regulation exist in systemic lupus erythematosus patients with distinct autoantibody specificities. J Clin Immunol *34*, 491-503.

Chen, J.M., Cooper, D.N., Ferec, C., Kehrer-Sawatzki, H., and Patrinos, G.P. (2010). Genomic rearrangements in inherited disease and cancer. Semin Cancer Biol *20*, 222-233.

Chen, L., Dahlstrom, J.E., Lee, S.H., and Rangasamy, D. (2012). Naturally occurring endo-siRNA silences LINE-1 retrotransposons in human cells through DNA methylation. Epigenetics *7*, 758-771.

Chen, W., Bocker, W., Brosius, J., and Tiedge, H. (1997a). Expression of neural BC200 RNA in human tumours. J Pathol *183*, 345-351.

Chen, W., Heierhorst, J., Brosius, J., and Tiedge, H. (1997b). Expression of neural BC1 RNA: induction in murine tumours. Eur J Cancer *33*, 288-292.

Cheon, D.J., and Orsulic, S. (2011). Mouse models of cancer. Annu Rev Pathol *6*, 95-119.

Chiu, Y.H., Macmillan, J.B., and Chen, Z.J. (2009). RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. Cell *138*, 576-591.

Choi, S.J., and Szoka, F.C. (2000). Fluorometric determination of deoxyribonuclease I activity with PicoGreen. Anal Biochem *281*, 95-97.

Chowdhury, D., Beresford, P.J., Zhu, P., Zhang, D., Sung, J.S., Demple, B., Perrino, F.W., and Lieberman, J. (2006). The exonuclease TREX1 is in the SET complex and acts in concert with NM23-H1 to degrade DNA during granzyme A-mediated cell death. Mol Cell *23*, 133-142.

Christmann, M., Tomicic, M.T., Aasland, D., Berdelle, N., and Kaina, B. (2010). Three prime exonuclease I (TREX1) is Fos/AP-1 regulated by genotoxic stress and protects against ultraviolet light and benzo(a)pyrene-induced DNA damage. Nucleic Acids Res *38*, 6418-6432.

ChunJiao, S., Huan, C., ChaoYang, X., and GuoMei, R. (2014). Uncovering the roles of miRNAs and their relationship with androgen receptor in prostate cancer. IUBMB Life *66*, 379-386.

Ciccia, A., and Elledge, S.J. (2010). The DNA damage response: making it safe to play with knives. Mol Cell *40*, 179-204.

Cros, E., Jordheim, L., Dumontet, C., and Galmarini, C.M. (2004). Problems related to resistance to cytarabine in acute myeloid leukemia. Leuk Lymphoma *45*, 1123-1132.

Crow, Y.J., Leitch, A., Hayward, B.E., Garner, A., Parmar, R., Griffith, E., Ali, M., Semple, C., Aicardi, J., Babul-Hirji, R., Baumann, C., Baxter, P., Bertini, E., Chandler, K.E., Chitayat, D., Cau, D., Dery, C., Fazzi, E., Goizet, C., King, M.D., Klepper, J., Lacombe, D., Lanzi, G., Lyall, H., Martinez-Frias, M.L., Mathieu, M., McKeown, C., Monier, A., Oade, Y., Quarrell, O.W., Rittey, C.D., Rogers, R.C.,

Sanchis, A., Stephenson, J.B., Tacke, U., Till, M., Tolmie, J.L., Tomlin, P., Voit, T., Weschke, B., Woods, C.G., Lebon, P., Bonthron, D.T., Ponting, C.P., and Jackson, A.P. (2006). Mutations in genes encoding ribonuclease H2 subunits cause Aicardi-Goutieres syndrome and mimic congenital viral brain infection. Nat Genet *38*, 910-916.

Croxford, J.L., Tang, M.L., Pan, M.F., Huang, C.W., Kamran, N., Phua, C.M., Chng, W.J., Ng, S.B., Raulet, D.H., and Gasser, S. (2013). ATM-dependent spontaneous regression of early Emu-myc-induced murine B-cell leukemia depends on natural killer and T cells. Blood *121*, 2512-2521.

Cunha, C., Carvalho, A., Esposito, A., Bistoni, F., and Romani, L. (2012). DAMP signaling in fungal infections and diseases. Front Immunol *3*, 286.

Dardenne, E., Pierredon, S., Driouch, K., Gratadou, L., Lacroix-Triki, M., Espinoza, M.P., Zonta, E., Germann, S., Mortada, H., Villemin, J.P., Dutertre, M., Lidereau, R., Vagner, S., and Auboeuf, D. (2012). Splicing switch of an epigenetic regulator by RNA helicases promotes tumor-cell invasiveness. Nat Struct Mol Biol *19*, 1139-1146.

Dardenne, E., Polay Espinoza, M., Fattet, L., Germann, S., Lambert, M.P., Neil, H., Zonta, E., Mortada, H., Gratadou, L., Deygas, M., Chakrama, F.Z., Samaan, S., Desmet, F.O., Tranchevent, L.C., Dutertre, M., Rimokh, R., Bourgeois, C.F., and Auboeuf, D. (2014). RNA Helicases DDX5 and DDX17 Dynamically Orchestrate Transcription, miRNA, and Splicing Programs in Cell Differentiation. Cell Rep.

Das, S.K., Sokhi, U.K., Bhutia, S.K., Azab, B., Su, Z.Z., Sarkar, D., and Fisher, P.B. (2010). Human polynucleotide phosphorylase selectively and preferentially degrades microRNA-221 in human melanoma cells. Proc Natl Acad Sci U S A *107*, 11948-11953.

David, N.A. (1972). The pharmacology of dimethyl sulfoxide. Annu Rev Pharmacol *12*, 353-374.

de Parseval, N., Lazar, V., Casella, J.F., Benit, L., and Heidmann, T. (2003). Survey of human genes of retroviral origin: identification and transcriptome of the genes with coding capacity for complete envelope proteins. J Virol *77*, 10414-10422.

Deigendesch, N., Koch-Nolte, F., and Rothenburg, S. (2006). ZBP1 subcellular localization and association with stress granules is controlled by its Z-DNA binding domains. Nucleic Acids Res *34*, 5007-5020.

Dieci, G., Conti, A., Pagano, A., and Carnevali, D. (2013). Identification of RNA polymerase III-transcribed genes in eukaryotic genomes. Biochim Biophys Acta *1829*, 296-305.

Dieci, G., Fiorino, G., Castelnuovo, M., Teichmann, M., and Pagano, A. (2007). The expanding RNA polymerase III transcriptome. Trends Genet *23*, 614-622.

Dragan, A.I., Casas-Finet, J.R., Bishop, E.S., Strouse, R.J., Schenerman, M.A., and Geddes, C.D. (2010). Characterization of PicoGreen interaction with dsDNA and the origin of its fluorescence enhancement upon binding. Biophys J *99*, 3010-3019.

Dvorakova, M., Karafiat, V., Pajer, P., Kluzakova, E., Jarkovska, K., Pekova, S., Krutilkova, L., and Dvorak, M. (2013). DNA released by leukemic cells contributes to the disruption of the bone marrow microenvironment. Oncogene *32*, 5201-5209.

Eddy, J., and Maizels, N. (2008). Conserved elements with potential to form polymorphic G-quadruplex structures in the first intron of human genes. Nucleic Acids Res *36*, 1321-1333.

Egawa, S., Uchida, T., Suyama, K., Wang, C., Ohori, M., Irie, S., Iwamura, M., and Koshiba, K. (1995). Genomic instability of microsatellite repeats in prostate cancer: relationship to clinicopathological variables. Cancer Res *55*, 2418-2421.

Felsher, D.W., and Bishop, J.M. (1999). Transient excess of MYC activity can elicit genomic instability and tumorigenesis. Proc Natl Acad Sci U S A *96*, 3940-3944.

Feng, Q., Moran, J.V., Kazazian, H.H., Jr., and Boeke, J.D. (1996). Human L1 retrotransposon encodes a conserved endonuclease required for retrotransposition. Cell *87*, 905-916.

Franchitto, A., and Pichierri, P. (2011). Understanding the molecular basis of common fragile sites instability: role of the proteins involved in the recovery of stalled replication forks. Cell Cycle *10*, 4039-4046.

Frank, P., Albert, S., Cazenave, C., and Toulme, J.J. (1994). Purification and characterization of human ribonuclease HII. Nucleic Acids Res *22*, 5247-5254.

Fredlund, J., and Enninga, J. (2014). Cytoplasmic access by intracellular bacterial pathogens. Trends Microbiol *22*, 128-137.

Fukuda, T., Yamagata, K., Fujiyama, S., Matsumoto, T., Koshida, I., Yoshimura, K., Mihara, M., Naitou, M., Endoh, H., Nakamura, T., Akimoto, C., Yamamoto, Y., Katagiri, T., Foulds, C., Takezawa, S., Kitagawa, H., Takeyama, K., O'Malley, B.W., and Kato, S. (2007). DEAD-box RNA helicase subunits of the Drosha complex are required for processing of rRNA and a subset of microRNAs. Nat Cell Biol *9*, 604-611.

Fuller-Pace, F.V. (2013). The DEAD box proteins DDX5 (p68) and DDX17 (p72): Multi-tasking transcriptional regulators. Biochim Biophys Acta *1829*, 756-763.

Fuller-Pace, F.V., and Moore, H.C. (2011). RNA helicases p68 and p72: multifunctional proteins with important implications for cancer development. Future Oncol *7*, 239-251.

Furuta, Y., Gowen, B.B., Takahashi, K., Shiraki, K., Smee, D.F., and Barnard, D.L. (2013). Favipiravir (T-705), a novel viral RNA polymerase inhibitor. Antiviral Res *100*, 446-454.

Gacy, A.M., Goellner, G., Juranic, N., Macura, S., and McMurray, C.T. (1995). Trinucleotide repeats that expand in human disease form hairpin structures in vitro. Cell *81*, 533-540.

Gao, D., Wu, J., Wu, Y.T., Du, F., Aroh, C., Yan, N., Sun, L., and Chen, Z.J. (2013). Cyclic GMP-AMP synthase is an innate immune sensor of HIV and other retroviruses. Science *341*, 903-906.

Gao, X., Ge, L., Shao, J., Su, C., Zhao, H., Saarikettu, J., Yao, X., Yao, Z., Silvennoinen, O., and Yang, J. (2010). Tudor-SN interacts with and co-localizes with G3BP in stress granules under stress conditions. FEBS Lett *584*, 3525-3532.

Gasior, S.L., Wakeman, T.P., Xu, B., and Deininger, P.L. (2006). The human LINE-1 retrotransposon creates DNA double-strand breaks. J Mol Biol *357*, 1383-1393.

Gasser, S., Orsulic, S., Brown, E.J., and Raulet, D.H. (2005). The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. Nature *436*, 1186-1190.

Georges, S.A., Biery, M.C., Kim, S.Y., Schelter, J.M., Guo, J., Chang, A.N., Jackson, A.L., Carleton, M.O., Linsley, P.S., Cleary, M.A., and Chau, B.N. (2008). Coordinated regulation of cell cycle transcripts by p53-Inducible microRNAs, miR-192 and miR-215. Cancer Res *68*, 10105-10112.

Germann, S., Gratadou, L., Zonta, E., Dardenne, E., Gaudineau, B., Fougere, M., Samaan, S., Dutertre, M., Jauliac, S., and Auboeuf, D. (2012). Dual role of the ddx5/ddx17 RNA helicases in the control of the pro-migratory NFAT5 transcription factor. Oncogene *31*, 4536-4549.

Ginno, P.A., Lott, P.L., Christensen, H.C., Korf, I., and Chedin, F. (2012). R-loop formation is a distinctive characteristic of unmethylated human CpG island promoters. Mol Cell *45*, 814-825.

Gjidoda, A., and Henry, R.W. (2013). RNA polymerase III repression by the retinoblastoma tumor suppressor protein. Biochim Biophys Acta *1829*, 385-392.

Gmeiner, W.H., Skradis, A., Pon, R.T., and Liu, J. (1998). Cytarabine-induced destabilization of a model Okazaki fragment. Nucleic Acids Res *26*, 2359-2365.

Goodrich, J.A., and Kugel, J.F. (2006). Non-coding-RNA regulators of RNA polymerase II transcription. Nature reviews. Molecular cell biology *7*, 612-616.

Hagan, C.R., and Rudin, C.M. (2007). DNA cleavage and Trp53 differentially affect SINE transcription. Genes Chromosomes Cancer *46*, 248-260.

Hamberg, A., Ringler, S., and Krakowka, S. (2007). A novel method for the detection of porcine circovirus type 2 replicative double stranded viral DNA and nonreplicative single stranded viral DNA in tissue sections. J Vet Diagn Invest *19*, 135-141.

Hammond-Thelin, L.A., Thomas, M.B., Iwasaki, M., Abbruzzese, J.L., Lassere, Y., Meyers, C.A., Hoff, P., de Bono, J., Norris, J., Matsushita, H., Mita, A., and Rowinsky, E.K. (2012). Phase I and pharmacokinetic study of 3'-C-ethynylcytidine (TAS-106), an inhibitor of RNA polymerase I, II and III,in patients with advanced solid malignancies. Invest New Drugs *30*, 316-326.

Hamperl, S., and Cimprich, K.A. (2014). The contribution of co-transcriptional RNA:DNA hybrid structures to DNA damage and genome instability. DNA Repair (Amst) *19*, 84-94.

Han, J., Lee, Y., Yeom, K.H., Kim, Y.K., Jin, H., and Kim, V.N. (2004). The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev *18*, 3016-3027.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.

Haq, I., Ladbury, J.E., Chowdhry, B.Z., Jenkins, T.C., and Chaires, J.B. (1997). Specific binding of hoechst 33258 to the d(CGCAAATTTGCG)2 duplex: calorimetric and spectroscopic studies. J Mol Biol *271*, 244-257.

Harrington, C., and Perrino, F.W. (1995). Initiation of RNA-primed DNA synthesis in vitro by DNA polymerase alpha-primase. Nucleic Acids Res *23*, 1003-1009.

Harris, A.W., Pinkert, C.A., Crawford, M., Langdon, W.Y., Brinster, R.L., and Adams, J.M. (1988). The E mu-myc transgenic mouse. A model for high-incidence spontaneous lymphoma and leukemia of early B cells. J Exp Med *167*, 353-371.

He, L., Thomson, J.M., Hemann, M.T., Hernando-Monge, E., Mu, D., Goodson, S., Powers, S., Cordon-Cardo, C., Lowe, S.W., Hannon, G.J., and Hammond, S.M. (2005). A microRNA polycistron as a potential human oncogene. Nature *435*, 828-833.

Hedges, D.J., and Deininger, P.L. (2007). Inviting instability: Transposable elements, double-strand breaks, and the maintenance of genome integrity. Mutat Res *616*, 46-59.

Helgason, E., Phung, Q.T., and Dueber, E.C. (2013). Recent insights into the complexity of Tank-binding kinase 1 signaling networks: the emerging role of cellular localization in the activation and substrate specificity of TBK1. FEBS Lett *587*, 1230-1237.

Hemmi, H., Takeuchi, O., Sato, S., Yamamoto, M., Kaisho, T., Sanjo, H., Kawai, T., Hoshino, K., Takeda, K., and Akira, S. (2004). The roles of two IkappaB kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection. J Exp Med *199*, 1641-1650.

Hesselstrand, R., Scheja, A., and Wuttge, D.M. (2012). Scleroderma renal crisis in a Swedish systemic sclerosis cohort: survival, renal outcome, and RNA polymerase III antibodies as a risk factor. Scand J Rheumatol *41*, 39-43.

Hiller, B., Achleitner, M., Glage, S., Naumann, R., Behrendt, R., and Roers, A. (2012). Mammalian RNase H2 removes ribonucleotides from DNA to maintain genome integrity. J Exp Med *209*, 1419-1426.

Ho, C.C., Siu, W.Y., Lau, A., Chan, W.M., Arooz, T., and Poon, R.Y. (2006). Stalled replication induces p53 accumulation through distinct mechanisms from DNA damage checkpoint pathways. Cancer Res *66*, 2233-2241.

Hoeijmakers, J.H. (2009). DNA damage, aging, and cancer. N Engl J Med *361*, 1475-1485.

Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K.K., Schlee, M., Endres, S., and Hartmann, G. (2006). 5'-Triphosphate RNA is the ligand for RIG-I. Science *314*, 994-997.

Hornung, V., and Latz, E. (2010). Intracellular DNA recognition. Nat Rev Immunol *10*, 123-130.

Horton, N.C., and Finzel, B.C. (1996). The structure of an RNA/DNA hybrid: a substrate of the ribonuclease activity of HIV-1 reverse transcriptase. J Mol Biol *264*, 521-533.

Hu, Z., Zhang, A., Storz, G., Gottesman, S., and Leppla, S.H. (2006). An antibody-based microarray assay for small RNA detection. Nucleic Acids Res *34*, e52.

Huang, J.C., Svoboda, D.L., Reardon, J.T., and Sancar, A. (1992). Human nucleotide excision nuclease removes thymine dimers from DNA by incising the 22nd phosphodiester bond 5' and the 6th phosphodiester bond 3' to the photodimer. Proc Natl Acad Sci U S A *89*, 3664-3668.

Iben, J.R., Mazeika, J.K., Hasson, S., Rijal, K., Arimbasseri, A.G., Russo, A.N., and Maraia, R.J. (2011). Point mutations in the Rpb9-homologous domain of Rpc11 that impair transcription termination by RNA polymerase III. Nucleic Acids Res *39*, 6100-6113.

Ishii, K.J., Coban, C., Kato, H., Takahashi, K., Torii, Y., Takeshita, F., Ludwig, H., Sutter, G., Suzuki, K., Hemmi, H., Sato, S., Yamamoto, M., Uematsu, S., Kawai, T., Takeuchi, O., and Akira, S. (2006). A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. Nat Immunol *7*, 40-48.

Ishii, K.J., Kawagoe, T., Koyama, S., Matsui, K., Kumar, H., Kawai, T., Uematsu, S., Takeuchi, O., Takeshita, F., Coban, C., and Akira, S. (2008). TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. Nature *451*, 725-729.

Ishikawa, H., and Barber, G.N. (2011). The STING pathway and regulation of innate immune signaling in response to DNA pathogens. Cell Mol Life Sci *68*, 1157-1165.

Ivanov, S., Dragoi, A.M., Wang, X., Dallacosta, C., Louten, J., Musco, G., Sitia, G., Yap, G.S., Wan, Y., Biron, C.A., Bianchi, M.E., Wang, H., and Chu, W.M. (2007). A novel role for HMGB1 in TLR9-mediated inflammatory responses to CpG-DNA. Blood *110*, 1970-1981.

Jaehning, J.A., and Roeder, R.G. (1977). Transcription of specific adenovirus genes in isolated nuclei by exogenous RNA polymerases. J Biol Chem *252*, 8753-8761.

Jaiswal, M., LaRusso, N.F., Burgart, L.J., and Gores, G.J. (2000). Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide-dependent mechanism. Cancer Res *60*, 184-190.

Jamieson, G.P., Snook, M.B., and Wiley, J.S. (1990). Saturation of intracellular cytosine arabinoside triphosphate accumulation in human leukemic blast cells. Leuk Res *14*, 475-479.

Jensen, S.B., and Paludan, S.R. (2014). Sensing the hybrid--a novel PAMP for TLR9. Embo J.

Johnson, S.A., Dubeau, L., and Johnson, D.L. (2008). Enhanced RNA polymerase III-dependent transcription is required for oncogenic transformation. J Biol Chem *283*, 19184-19191.

Jounai, N., Kobiyama, K., Takeshita, F., and Ishii, K.J. (2012). Recognition of damage-associated molecular patterns related to nucleic acids during inflammation and vaccination. Front Cell Infect Microbiol *2*, 168.

Kailasan Vanaja, S., Rathinam, V.A., Atianand, M.K., Kalantari, P., Skehan, B., Fitzgerald, K.A., and Leong, J.M. (2014). Bacterial RNA:DNA hybrids are activators of the NLRP3 inflammasome. Proc Natl Acad Sci U S A.

Kambach, C., Walke, S., and Nagai, K. (1999). Structure and assembly of the spliceosomal small nuclear ribonucleoprotein particles. Curr Opin Struct Biol *9*, 222-230.

Kasahara, M., Clikeman, J.A., Bates, D.B., and Kogoma, T. (2000). RecA protein-dependent R-loop formation in vitro. Genes Dev *14*, 360-365.

Kashii, Y., Giorda, R., Herberman, R.B., Whiteside, T.L., and Vujanovic, N.L. (1999). Constitutive expression and role of the TNF family ligands in apoptotic killing of tumor cells by human NK cells. J Immunol *163*, 5358-5366.

Kavanagh, D., Spitzer, D., Kothari, P.H., Shaikh, A., Liszewski, M.K., Richards, A., and Atkinson, J.P. (2008). New roles for the major human 3'-5' exonuclease TREX1 in human disease. Cell Cycle *7*, 1718-1725.

Kawai, S., and Amano, A. (2012). BRCA1 regulates microRNA biogenesis via the DROSHA microprocessor complex. J Cell Biol *197*, 201-208.

Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., Ishii, K.J., Takeuchi, O., and Akira, S. (2005). IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat Immunol *6*, 981-988.

Keller, W., and Crouch, R. (1972). Degradation of DNA RNA hybrids by ribonuclease H and DNA polymerases of cellular and viral origin. Proc Natl Acad Sci U S A *69*, 3360-3364.

Kim, T., Kim, T.Y., Song, Y.H., Min, I.M., Yim, J., and Kim, T.K. (1999). Activation of interferon regulatory factor 3 in response to DNA-damaging agents. J Biol Chem *274*, 30686-30689.

Kinoshita, E., van der Linden, E., Sanchez, H., and Wyman, C. (2009). RAD50, an SMC family member with multiple roles in DNA break repair: how does ATP affect function? Chromosome Res *17*, 277-288.

Kireeva, M.L., Komissarova, N., Waugh, D.S., and Kashlev, M. (2000). The 8-nucleotide-long RNA:DNA hybrid is a primary stability determinant of the RNA polymerase II elongation complex. J Biol Chem *275*, 6530-6536.

Kloosterman, W.P., and Plasterk, R.H. (2006). The diverse functions of microRNAs in animal development and disease. Dev Cell *11*, 441-450.

Kobiyama, K., Takeshita, F., Jounai, N., Sakaue-Sawano, A., Miyawaki, A., Ishii, K.J., Kawai, T., Sasaki, S., Hirano, H., Ishii, N., Okuda, K., and Suzuki, K. (2010). Extrachromosomal histone H2B mediates innate antiviral immune responses induced by intracellular double-stranded DNA. J Virol *84*, 822-832.

Komissarova, N., Becker, J., Solter, S., Kireeva, M., and Kashlev, M. (2002). Shortening of RNA:DNA hybrid in the elongation complex of RNA polymerase is a prerequisite for transcription termination. Mol Cell *10*, 1151-1162.

Komissarova, N., and Kashlev, M. (1998). Functional topography of nascent RNA in elongation intermediates of RNA polymerase. Proc Natl Acad Sci U S A *95*, 14699-14704.

Kramerov, D.A., and Vassetzky, N.S. (2005). Short retroposons in eukaryotic genomes. Int Rev Cytol *247*, 165-221.

Krynetskaia, N.F., Phadke, M.S., Jadhav, S.H., and Krynetskiy, E.Y. (2009). Chromatin-associated proteins HMGB1/2 and PDIA3 trigger cellular response to chemotherapy-induced DNA damage. Mol Cancer Ther *8*, 864-872.

Kuhn, C.D., Geiger, S.R., Baumli, S., Gartmann, M., Gerber, J., Jennebach, S., Mielke, T., Tschochner, H., Beckmann, R., and Cramer, P. (2007). Functional architecture of RNA polymerase I. Cell *131*, 1260-1272.

Kumar, M.S., Lu, J., Mercer, K.L., Golub, T.R., and Jacks, T. (2007). Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nat Genet *39*, 673-677.

Kunkel, G.R., Maser, R.L., Calvet, J.P., and Pederson, T. (1986). U6 small nuclear RNA is transcribed by RNA polymerase III. Proc Natl Acad Sci U S A *83*, 8575-8579.

Kuzminov, A. (2001). Single-strand interruptions in replicating chromosomes cause double-strand breaks. Proc Natl Acad Sci U S A *98*, 8241-8246.

Lam, A.R., Le Bert, N., Ho, S.S., Shen, Y.J., Tang, M.L., Xiong, G.M., Croxford, J.L., Koo, C.X., Ishii, K.J., Akira, S., Raulet, D.H., and Gasser, S. (2014a). RAE1 Ligands for the NKG2D Receptor Are Regulated by STING-Dependent DNA Sensor Pathways in Lymphoma. Cancer Res.

Lam, E., Stein, S., and Falck-Pedersen, E. (2014b). Adenovirus detection by the cGAS/STING/TBK1 DNA sensing cascade. J Virol *88*, 974-981.

Lam, E.Y., Beraldi, D., Tannahill, D., and Balasubramanian, S. (2013). G-quadruplex structures are stable and detectable in human genomic DNA. Nat Commun *4*, 1796.

Langdon, W.Y., Harris, A.W., Cory, S., and Adams, J.M. (1986). The c-myc oncogene perturbs B lymphocyte development in E-mu-myc transgenic mice. Cell *47*, 11-18.

Lee, Y., Kim, M., Han, J., Yeom, K.H., Lee, S., Baek, S.H., and Kim, V.N. (2004). MicroRNA genes are transcribed by RNA polymerase II. Embo J *23*, 4051-4060.

Leek, R.D., Landers, R., Fox, S.B., Ng, F., Harris, A.L., and Lewis, C.E. (1998). Association of tumour necrosis factor alpha and its receptors with thymidine phosphorylase expression in invasive breast carcinoma. Br J Cancer *77*, 2246-2251.

Li, J., Mbow, M.L., Sun, L., Li, L., Yang, G., Griswold, D.E., Schantz, A., Shealy, D.J., Goletz, T.J., Wan, J., and Peritt, D. (2004). Induction of dendritic cell maturation by IL-18. Cell Immunol *227*, 103-108.

Li, X., Shu, C., Yi, G., Chaton, C.T., Shelton, C.L., Diao, J., Zuo, X., Kao, C.C., Herr, A.B., and Li, P. (2013). Cyclic GMP-AMP synthase is activated by double-stranded DNA-induced oligomerization. Immunity *39*, 1019-1031.

Ligasova, A., Strunin, D., and Koberna, K. (2013). A New Method of the Visualization of the Double-Stranded Mitochondrial and Nuclear DNA. PLoS One *8*, e66864.

Lin, C., Yang, L., Yang, J.J., Huang, Y., and Liu, Z.R. (2005). ATPase/helicase activities of p68 RNA helicase are required for pre-mRNA splicing but not for assembly of the spliceosome. Mol Cell Biol *25*, 7484-7493.

Lindahl, T., and Barnes, D.E. (2000). Repair of endogenous DNA damage. Cold Spring Harb Symp Quant Biol *65*, 127-133.

Lindahl, T., Barnes, D.E., Yang, Y.G., and Robins, P. (2009). Biochemical properties of mammalian TREX1 and its association with DNA replication and inherited inflammatory disease. Biochem Soc Trans *37*, 535-538.

Liu, M.F., Wu, X.P., Wang, X.L., Yu, Y.L., Wang, W.F., Chen, Q.J., Boireau, P., and Liu, M.Y. (2008). The functions of Deoxyribonuclease II in immunity and development. DNA Cell Biol *27*, 223-228.

Lord, C.J., and Ashworth, A. (2012). The DNA damage response and cancer therapy. Nature *481*, 287-294.

Lorenzen, K., Vannini, A., Cramer, P., and Heck, A.J. (2007). Structural biology of RNA polymerase III: mass spectrometry elucidates subcomplex architecture. Structure *15*, 1237-1245.

Macfarlane, L.A., and Murphy, P.R. (2010). MicroRNA: Biogenesis, Function and Role in Cancer. Curr Genomics *11*, 537-561.

Marichal, T., Ohata, K., Bedoret, D., Mesnil, C., Sabatel, C., Kobiyama, K., Lekeux, P., Coban, C., Akira, S., Ishii, K.J., Bureau, F., and Desmet, C.J. (2011). DNA released from dying host cells mediates aluminum adjuvant activity. Nat Med *17*, 996-1002.

Marshall, L., and White, R.J. (2008). Non-coding RNA production by RNA polymerase III is implicated in cancer. Nat Rev Cancer *8*, 911-914.

Matsuoka, S., Ballif, B.A., Smogorzewska, A., McDonald, E.R., 3rd, Hurov, K.E., Luo, J., Bakalarski, C.E., Zhao, Z., Solimini, N., Lerenthal, Y., Shiloh, Y., Gygi, S.P., and Elledge, S.J. (2007). ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science *316*, 1160-1166.

Medzhitov, R. (2008). Origin and physiological roles of inflammation. Nature *454*, 428-435.

Meyer, N., and Penn, L.Z. (2008). Reflecting on 25 years with MYC. Nat Rev Cancer *8*, 976-990.

Miyahira, A.K., Shahangian, A., Hwang, S., Sun, R., and Cheng, G. (2009). TANK-binding kinase-1 plays an important role during in vitro and in vivo type I IFN responses to DNA virus infections. J Immunol *182*, 2248-2257.

Mizushima, N. (2007). Autophagy: process and function. Genes Dev *21*, 2861-2873.

Mogensen, T.H. (2009). Pathogen recognition and inflammatory signaling in innate immune defenses. Clin Microbiol Rev *22*, 240-273, Table of Contents.

Moinzadeh, P., Fonseca, C., Hellmich, M., Shah, A.A., Chighizola, C., Denton, C.P., and Ong, V.H. (2014). Association of anti-RNA polymerase III autoantibodies and cancer in scleroderma. Arthritis Res Ther *16*, R53.

Moran, J.V., Holmes, S.E., Naas, T.P., DeBerardinis, R.J., Boeke, J.D., and Kazazian, H.H., Jr. (1996). High frequency retrotransposition in cultured mammalian cells. Cell *87*, 917-927.

Morita, M., Stamp, G., Robins, P., Dulic, A., Rosewell, I., Hrivnak, G., Daly, G., Lindahl, T., and Barnes, D.E. (2004). Gene-targeted mice lacking the Trex1 (DNase III) 3'-->5' DNA exonuclease develop inflammatory myocarditis. Mol Cell Biol *24*, 6719-6727.

Musinova, Y.R., Lisitsyna, O.M., Golyshev, S.A., Tuzhikov, A.I., Polyakov, V.Y., and Sheval, E.V. (2011). Nucleolar localization/retention signal is responsible for transient accumulation of histone H2B in the nucleolus through electrostatic interactions. Biochim Biophys Acta *1813*, 27-38.

Nakama, M., Kawakami, K., Kajitani, T., Urano, T., and Murakami, Y. (2012). DNA-RNA hybrid formation mediates RNAi-directed heterochromatin formation. Genes Cells *17*, 218-233.

Nakhaei, P., Hiscott, J., and Lin, R. (2010). STING-ing the antiviral pathway. J Mol Cell Biol *2*, 110-112.

Napirei, M., Karsunky, H., Zevnik, B., Stephan, H., Mannherz, H.G., and Moroy, T. (2000). Features of systemic lupus erythematosus in Dnase1-deficient mice. Nat Genet *25*, 177-181.

Nicol, S.M., Bray, S.E., Derek Black, H., Lorimore, S.A., Wright, E.G., Lane, D.P., Meek, D.W., Coates, P.J., and Fuller-Pace, F.V. (2012). The RNA helicase p68 (DDX5) is selectively required for the induction of p53-dependent p21 expression and cell-cycle arrest after DNA damage. Oncogene.

Nowotny, M., Cerritelli, S.M., Ghirlando, R., Gaidamakov, S.A., Crouch, R.J., and Yang, W. (2008). Specific recognition of RNA/DNA hybrid and enhancement of human RNase H1 activity by HBD. Embo J *27*, 1172-1181.

O'Donnell, K.A., Wentzel, E.A., Zeller, K.I., Dang, C.V., and Mendell, J.T. (2005). c-Myc-regulated microRNAs modulate E2F1 expression. Nature *435*, 839-843.

Okabe, Y., Kawane, K., Akira, S., Taniguchi, T., and Nagata, S. (2005). Toll-like receptor-independent gene induction program activated by mammalian DNA escaped from apoptotic DNA degradation. J Exp Med *202*, 1333-1339.

Okada, N., Lin, C.P., Ribeiro, M.C., Biton, A., Lai, G., He, X., Bu, P., Vogel, H., Jablons, D.M., Keller, A.C., Wilkinson, J.E., He, B., Speed, T.P., and He, L. (2014). A positive feedback between p53 and miR-34 miRNAs mediates tumor suppression. Genes Dev *28*, 438-450.

Onomoto, K., Jogi, M., Yoo, J.S., Narita, R., Morimoto, S., Takemura, A., Sambhara, S., Kawaguchi, A., Osari, S., Nagata, K., Matsumiya, T., Namiki, H., Yoneyama, M., and Fujita, T. (2012). Critical role of an antiviral stress granule containing RIG-I and PKR in viral detection and innate immunity. PLoS One *7*, e43031.

Oppenheim, J.J., and Yang, D. (2005). Alarmins: chemotactic activators of immune responses. Curr Opin Immunol *17*, 359-365.

Orom, U.A., Kauppinen, S., and Lund, A.H. (2006). LNA-modified oligonucleotides mediate specific inhibition of microRNA function. Gene *372*, 137-141.

Ozsolak, F., Poling, L.L., Wang, Z., Liu, H., Liu, X.S., Roeder, R.G., Zhang, X., Song, J.S., and Fisher, D.E. (2008). Chromatin structure analyses identify miRNA promoters. Genes Dev *22*, 3172-3183.

Pagano, A., Castelnuovo, M., Tortelli, F., Ferrari, R., Dieci, G., and Cancedda, R. (2007). New small nuclear RNA gene-like transcriptional units as sources of regulatory transcripts. PLoS Genet *3*, e1.

Palucka, A.K., Blanck, J.P., Bennett, L., Pascual, V., and Banchereau, J. (2005). Cross-regulation of TNF and IFN-alpha in autoimmune diseases. Proc Natl Acad Sci U S A *102*, 3372-3377.

Pamment, J., Ramsay, E., Kelleher, M., Dornan, D., and Ball, K.L. (2002). Regulation of the IRF-1 tumour modifier during the response to genotoxic stress involves an ATM-dependent signalling pathway. Oncogene *21*, 7776-7785.

Pedrali-Noy, G., and Spadari, S. (1979). Effect of aphidicolin on viral and human DNA polymerases. Biochem Biophys Res Commun *88*, 1194-1202.

Perry, A.K., Chow, E.K., Goodnough, J.B., Yeh, W.C., and Cheng, G. (2004). Differential requirement for TANK-binding kinase-1 in type I interferon responses to toll-like receptor activation and viral infection. J Exp Med *199*, 1651-1658.

Peterson, J.M., Feeback, K.D., Baas, J.H., and Pizza, F.X. (2006). Tumor necrosis factor-alpha promotes the accumulation of neutrophils and macrophages in skeletal muscle. J Appl Physiol (1985) *101*, 1394-1399.

Phillips, D.D., Garboczi, D.N., Singh, K., Hu, Z., Leppla, S.H., and Leysath, C.E. (2013). The sub-nanomolar binding of DNA-RNA hybrids by the single-chain Fv fragment of antibody S9.6. J Mol Recognit *26*, 376-381.

Ponicsan, S.L., Kugel, J.F., and Goodrich, J.A. (2010). Genomic gems: SINE RNAs regulate mRNA production. Curr Opin Genet Dev *20*, 149-155.

Pothof, J., Verkaik, N.S., Hoeijmakers, J.H., and van Gent, D.C. (2009). MicroRNA responses and stress granule formation modulate the DNA damage response. Cell Cycle *8*, 3462-3468.

Prevost-Blondel, A., Roth, E., Rosenthal, F.M., and Pircher, H. (2000). Crucial role of TNF-alpha in CD8 T cell-mediated elimination of 3LL-A9 Lewis lung carcinoma cells in vivo. J Immunol *164*, 3645-3651.

Pritchard, C.C., Kroh, E., Wood, B., Arroyo, J.D., Dougherty, K.J., Miyaji, M.M., Tait, J.F., and Tewari, M. (2012). Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies. Cancer Prev Res (Phila) *5*, 492-497.

Pusapati, R.V., Rounbehler, R.J., Hong, S., Powers, J.T., Yan, M., Kiguchi, K., McArthur, M.J., Wong, P.K., and Johnson, D.G. (2006). ATM promotes apoptosis and suppresses tumorigenesis in response to Myc. Proc Natl Acad Sci U S A *103*, 1446-1451.

Reijns, M.A., Rabe, B., Rigby, R.E., Mill, P., Astell, K.R., Lettice, L.A., Boyle, S., Leitch, A., Keighren, M., Kilanowski, F., Devenney, P.S., Sexton, D., Grimes, G., Holt, I.J., Hill, R.E., Taylor, M.S., Lawson, K.A., Dorin, J.R., and Jackson, A.P. (2012). Enzymatic removal of ribonucleotides from DNA is essential for mammalian genome integrity and development. Cell *149*, 1008-1022.

Reimann, M., Loddenkemper, C., Rudolph, C., Schildhauer, I., Teichmann, B., Stein, H., Schlegelberger, B., Dorken, B., and Schmitt, C.A. (2007). The Myc-evoked DNA damage response accounts for treatment resistance in primary lymphomas in vivo. Blood *110*, 2996-3004.

Rigby, R.E., Webb, L.M., Mackenzie, K.J., Li, Y., Leitch, A., Reijns, M.A., Lundie, R.J., Revuelta, A., Davidson, D.J., Diebold, S., Modis, Y., Macdonald, A.S., and Jackson, A.P. (2014). RNA:DNA hybrids are a novel molecular pattern sensed by TLR9. Embo J.

Rodier, F., Coppe, J.P., Patil, C.K., Hoeijmakers, W.A., Munoz, D.P., Raza, S.R., Freund, A., Campeau, E., Davalos, A.R., and Campisi, J. (2009). Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. Nat Cell Biol *11*, 973-979.

Rodriguez, R., Miller, K.M., Forment, J.V., Bradshaw, C.R., Nikan, M., Britton, S., Oelschlaegel, T., Xhemalce, B., Balasubramanian, S., and Jackson, S.P. (2012). Small-molecule-induced DNA damage identifies alternative DNA structures in human genes. Nat Chem Biol *8*, 301-310.

Rodriguez-Rocha, H., Garcia-Garcia, A., Panayiotidis, M.I., and Franco, R. (2011). DNA damage and autophagy. Mutat Res *711*, 158-166.

Rush, M.G., and Misra, R. (1985). Extrachromosomal DNA in eucaryotes. Plasmid *14*, 177-191.

Rychlik, M.P., Chon, H., Cerritelli, S.M., Klimek, P., Crouch, R.J., and Nowotny, M. (2010). Crystal structures of RNase H2 in complex with nucleic acid reveal the mechanism of RNA-DNA junction recognition and cleavage. Mol Cell *40*, 658-670.

Sampson, V.B., Rong, N.H., Han, J., Yang, Q., Aris, V., Soteropoulos, P., Petrelli, N.J., Dunn, S.P., and Krueger, L.J. (2007). MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells. Cancer Res *67*, 9762-9770.

Schlee, M., Roth, A., Hornung, V., Hagmann, C.A., Wimmenauer, V., Barchet, W., Coch, C., Janke, M., Mihailovic, A., Wardle, G., Juranek, S., Kato, H., Kawai, T., Poeck, H., Fitzgerald, K.A., Takeuchi, O., Akira, S., Tuschl, T., Latz, E., Ludwig, J., and Hartmann, G. (2009). Recognition of 5' triphosphate by RIG-I helicase requires

short blunt double-stranded RNA as contained in panhandle of negative-strand virus. Immunity *31*, 25-34.

Schulz, T.F. (2009). Cancer and viral infections in immunocompromised individuals. Int J Cancer *125*, 1755-1763.

Schwartz, L.B., Sklar, V.E., Jaehning, J.A., Weinmann, R., and Roeder, R.G. (1974). Isolation and partial characterization of the multiple forms of deoxyribonucleic acid-dependent ribonucleic acid polymerase in the mouse myeloma, MOPC 315. J Biol Chem *249*, 5889-5897.

Semizarov, D., Frost, L., Sarthy, A., Kroeger, P., Halbert, D.N., and Fesik, S.W. (2003). Specificity of short interfering RNA determined through gene expression signatures. Proc Natl Acad Sci U S A *100*, 6347-6352.

Seno, J.D., and Dynlacht, J.R. (2004). Intracellular redistribution and modification of proteins of the Mre11/Rad50/Nbs1 DNA repair complex following irradiation and heat-shock. J Cell Physiol *199*, 157-170.

Sentenac, A. (1985). Eukaryotic RNA polymerases. CRC Crit Rev Biochem *18*, 31-90.

Sharma, P., Azebi, S., England, P., Christensen, T., Moller-Larsen, A., Petersen, T., Batsche, E., and Muchardt, C. (2012). Citrullination of histone H3 interferes with HP1-mediated transcriptional repression. PLoS Genet *8*, e1002934.

Shaw, N.N., and Arya, D.P. (2008). Recognition of the unique structure of DNA:RNA hybrids. Biochimie *90*, 1026-1039.

Shen, W., Li, L., Pan, Q., Min, L., Dong, H., and Deng, J. (2006). Efficient and simple production of transgenic mice and rabbits using the new DMSO-sperm mediated exogenous DNA transfer method. Mol Reprod Dev *73*, 589-594.

Shilatifard, A. (1998). Factors regulating the transcriptional elongation activity of RNA polymerase II. FASEB J *12*, 1437-1446.

Shimada, K., Crother, T.R., Karlin, J., Dagvadorj, J., Chiba, N., Chen, S., Ramanujan, V.K., Wolf, A.J., Vergnes, L., Ojcius, D.M., Rentsendorj, A., Vargas, M., Guerrero, C., Wang, Y., Fitzgerald, K.A., Underhill, D.M., Town, T., and Arditi, M. (2012). Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. Immunity *36*, 401-414.

Sidman, C.L., Denial, T.M., Marshall, J.D., and Roths, J.B. (1993). Multiple mechanisms of tumorigenesis in E mu-myc transgenic mice. Cancer Res *53*, 1665-1669.

Singer, V.L., Jones, L.J., Yue, S.T., and Haugland, R.P. (1997). Characterization of PicoGreen reagent and development of a fluorescence-based solution assay for double-stranded DNA quantitation. Anal Biochem *249*, 228-238.

Singh, K., Carey, M., Saragosti, S., and Botchan, M. (1985). Expression of enhanced levels of small RNA polymerase III transcripts encoded by the B2 repeats in simian virus 40-transformed mouse cells. Nature *314*, 553-556.

Skourti-Stathaki, K., Proudfoot, N.J., and Gromak, N. (2011). Human senataxin resolves RNA/DNA hybrids formed at transcriptional pause sites to promote Xrn2-dependent termination. Mol Cell *42*, 794-805.

So, S., Davis, A.J., and Chen, D.J. (2009). Autophosphorylation at serine 1981 stabilizes ATM at DNA damage sites. J Cell Biol *187*, 977-990.

Spagnolo, L., Rivera-Calzada, A., Pearl, L.H., and Llorca, O. (2006). Three-dimensional structure of the human DNA-PKcs/Ku70/Ku80 complex assembled on DNA and its implications for DNA DSB repair. Mol Cell *22*, 511-519.

Spycher, C., Miller, E.S., Townsend, K., Pavic, L., Morrice, N.A., Janscak, P., Stewart, G.S., and Stucki, M. (2008). Constitutive phosphorylation of MDC1 physically links the MRE11-RAD50-NBS1 complex to damaged chromatin. J Cell Biol *181*, 227-240.

Srikrishna, G., and Freeze, H.H. (2009). Endogenous damage-associated molecular pattern molecules at the crossroads of inflammation and cancer. Neoplasia *11*, 615-628.

Stetson, D.B. (2012). Endogenous retroelements and autoimmune disease. Curr Opin Immunol *24*, 692-697.

Stetson, D.B., Ko, J.S., Heidmann, T., and Medzhitov, R. (2008). Trex1 prevents cell-intrinsic initiation of autoimmunity. Cell *134*, 587-598.

Stohr, N., Lederer, M., Reinke, C., Meyer, S., Hatzfeld, M., Singer, R.H., and Huttelmaier, S. (2006). ZBP1 regulates mRNA stability during cellular stress. J Cell Biol *175*, 527-534.

Sulli, G., Di Micco, R., and d'Adda di Fagagna, F. (2012). Crosstalk between chromatin state and DNA damage response in cellular senescence and cancer. Nat Rev Cancer *12*, 709-720.

Sun, L., Wu, J., Du, F., Chen, X., and Chen, Z.J. (2013). Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. Science *339*, 786-791.

Suzuki, H.I., Arase, M., Matsuyama, H., Choi, Y.L., Ueno, T., Mano, H., Sugimoto, K., and Miyazono, K. (2011). MCPIP1 ribonuclease antagonizes dicer and terminates microRNA biogenesis through precursor microRNA degradation. Mol Cell *44*, 424-436.

Takano, T., and Hatanaka, M. (1975a). DNA-RNA hybrid in cells infected by murine leukemia virus. Cold Spring Harb Symp Quant Biol *39 Pt 2*, 1009-1014.

Takano, T., and Hatanaka, M. (1975b). Fate of viral RNA of murine leukemia virus after infection. Proc Natl Acad Sci U S A *72*, 343-347.

Takaoka, A., Wang, Z., Choi, M.K., Yanai, H., Negishi, H., Ban, T., Lu, Y., Miyagishi, M., Kodama, T., Honda, K., Ohba, Y., and Taniguchi, T. (2007). DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. Nature *448*, 501-505.

Takeuchi, O., and Akira, S. (2010). Pattern recognition receptors and inflammation. Cell *140*, 805-820.

Tarsounas, M., Davies, A.A., and West, S.C. (2004). RAD51 localization and activation following DNA damage. Philos Trans R Soc Lond B Biol Sci *359*, 87-93.

Thakur, B.K., Zhang, H., Becker, A., Matei, I., Huang, Y., Costa-Silva, B., Zheng, Y., Hoshino, A., Brazier, H., Xiang, J., Williams, C., Rodriguez-Barrueco, R., Silva, J.M., Zhang, W., Hearn, S., Elemento, O., Paknejad, N., Manova-Todorova, K., Welte, K., Bromberg, J., Peinado, H., and Lyden, D. (2014). Double-stranded DNA in exosomes: a novel biomarker in cancer detection. Cell Res *24*, 766-769.

Thomson, J.M., Newman, M., Parker, J.S., Morin-Kensicki, E.M., Wright, T., and Hammond, S.M. (2006). Extensive post-transcriptional regulation of microRNAs and its implications for cancer. Genes Dev *20*, 2202-2207.

Tourriere, H., Chebli, K., Zekri, L., Courselaud, B., Blanchard, J.M., Bertrand, E., and Tazi, J. (2003). The RasGAP-associated endoribonuclease G3BP assembles stress granules. J Cell Biol *160*, 823-831.

Tsai, S.Y., Segovia, J.A., Chang, T.H., Morris, I.R., Berton, M.T., Tessier, P.A., Tardif, M.R., Cesaro, A., and Bose, S. (2014). DAMP molecule S100A9 acts as a molecular pattern to enhance inflammation during influenza A virus infection: role of DDX21-TRIF-TLR4-MyD88 pathway. PLoS Pathog *10*, e1003848.

Tsao, A., Hui, E.P., Juergens, R., Marur, S., Huat, T.E., Cher, G.B., Hong, R.L., Hong, W.K., and Chan, A.T. (2013). Phase II study of TAS-106 in patients with platinum-failure recurrent or metastatic head and neck cancer and nasopharyngeal cancer. Cancer Med *2*, 351-359.

Unterholzner, L., Keating, S.E., Baran, M., Horan, K.A., Jensen, S.B., Sharma, S., Sirois, C.M., Jin, T., Latz, E., Xiao, T.S., Fitzgerald, K.A., Paludan, S.R., and Bowie, A.G. (2010). IFI16 is an innate immune sensor for intracellular DNA. Nat Immunol *11*, 997-1004.

Vafa, O., Wade, M., Kern, S., Beeche, M., Pandita, T.K., Hampton, G.M., and Wahl, G.M. (2002). c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability. Mol Cell *9*, 1031-1044.

Valentine, R., and Smith, G.L. (2010). Inhibition of the RNA polymerase III-mediated dsDNA-sensing pathway of innate immunity by vaccinia virus protein E3. J Gen Virol *91*, 2221-2229.

van Kouwenhove, M., Kedde, M., and Agami, R. (2011). MicroRNA regulation by RNA-binding proteins and its implications for cancer. Nat Rev Cancer *11*, 644-656.

Van Nguyen, T., Puebla-Osorio, N., Pang, H., Dujka, M.E., and Zhu, C. (2007). DNA damage-induced cellular senescence is sufficient to suppress tumorigenesis: a mouse model. J Exp Med *204*, 1453-1461.

Veras, I., Rosen, E.M., and Schramm, L. (2009). Inhibition of RNA polymerase III transcription by BRCA1. J Mol Biol *387*, 523-531.

Vlachos, I.S., Kostoulas, N., Vergoulis, T., Georgakilas, G., Reczko, M., Maragkakis, M., Paraskevopoulou, M.D., Prionidis, K., Dalamagas, T., and Hatzigeorgiou, A.G. (2012). DIANA miRPath v.2.0: investigating the combinatorial effect of microRNAs in pathways. Nucleic Acids Res *40*, W498-504.

Volkman, H.E., and Stetson, D.B. (2014). The enemy within: endogenous retroelements and autoimmune disease. Nat Immunol *15*, 415-422.

Wahba, L., Amon, J.D., Koshland, D., and Vuica-Ross, M. (2011). RNase H and multiple RNA biogenesis factors cooperate to prevent RNA:DNA hybrids from generating genome instability. Mol Cell *44*, 978-988.

Wahba, L., Gore, S.K., and Koshland, D. (2013). The homologous recombination machinery modulates the formation of RNA-DNA hybrids and associated chromosome instability. Elife *2*, e00505.

Wan, G., Zhang, X., Langley, R.R., Liu, Y., Hu, X., Han, C., Peng, G., Ellis, L.M., Jones, S.N., and Lu, X. (2013). DNA-Damage-Induced Nuclear Export of Precursor MicroRNAs Is Regulated by the ATM-AKT Pathway. Cell Rep.

Wang, H., Bloom, O., Zhang, M., Vishnubhakat, J.M., Ombrellino, M., Che, J., Frazier, A., Yang, H., Ivanova, S., Borovikova, L., Manogue, K.R., Faist, E., Abraham, E., Andersson, J., Andersson, U., Molina, P.E., Abumrad, N.N., Sama, A., and Tracey, K.J. (1999). HMG-1 as a late mediator of endotoxin lethality in mice. Science *285*, 248-251.

Wang, Y., Huang, J.W., Li, M., Cavenee, W.K., Mitchell, P.S., Zhou, X., Tewari, M., Furnari, F.B., and Taniguchi, T. (2011). MicroRNA-138 modulates DNA damage response by repressing histone H2AX expression. Mol Cancer Res *9*, 1100-1111.

Wang, Y., Li, M., Stadler, S., Correll, S., Li, P., Wang, D., Hayama, R., Leonelli, L., Han, H., Grigoryev, S.A., Allis, C.D., and Coonrod, S.A. (2009). Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. J Cell Biol *184*, 205-213.

Wang, Y., and Taniguchi, T. (2013). MicroRNAs and DNA damage response: implications for cancer therapy. Cell Cycle *12*, 32-42.

Wang, Z., and Roeder, R.G. (1997). Three human RNA polymerase III-specific subunits form a subcomplex with a selective function in specific transcription initiation. Genes Dev *11*, 1315-1326.

Watson, J.D., and Crick, F.H. (1953). Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. Nature *171*, 737-738.

Weinmann, R., and Roeder, R.G. (1974). Role of DNA-dependent RNA polymerase 3 in the transcription of the tRNA and 5S RNA genes. Proc Natl Acad Sci U S A *71*, 1790-1794.

Whitcomb, J.M., and Hughes, S.H. (1992). Retroviral reverse transcription and integration: progress and problems. Annu Rev Cell Biol *8*, 275-306.

White, R.J. (2011). Transcription by RNA polymerase III: more complex than we thought. Nat Rev Genet *12*, 459-463.

Wiernik, P.H., Case, D.C., Jr., Periman, P.O., Arlin, Z.A., Weitberg, A.B., Ritch, P.S., and Todd, M.B. (1989). A multicenter trial of cytarabine plus idarubicin or daunorubicin as induction therapy for adult nonlymphocytic leukemia. Semin Oncol *16*, 25-29.

Williams, G.H., and Stoeber, K. (2012). The cell cycle and cancer. J Pathol *226*, 352-364.

Williams, M.C., Rouzina, I., and Bloomfield, V.A. (2002). Thermodynamics of DNA interactions from single molecule stretching experiments. Acc Chem Res *35*, 159-166.

Wimberly, H., Shee, C., Thornton, P.C., Sivaramakrishnan, P., Rosenberg, S.M., and Hastings, P.J. (2013). R-loops and nicks initiate DNA breakage and genome instability in non-growing Escherichia coli. Nat Commun *4*, 2115.

Winter, A.G., Sourvinos, G., Allison, S.J., Tosh, K., Scott, P.H., Spandidos, D.A., and White, R.J. (2000). RNA polymerase III transcription factor TFIIIC2 is overexpressed in ovarian tumors. Proc Natl Acad Sci U S A *97*, 12619-12624.

Wohrl, B.M., and Moelling, K. (1990). Interaction of HIV-1 ribonuclease H with polypurine tract containing RNA-DNA hybrids. Biochemistry *29*, 10141-10147.

Woiwode, A., Johnson, S.A., Zhong, S., Zhang, C., Roeder, R.G., Teichmann, M., and Johnson, D.L. (2008). PTEN represses RNA polymerase III-dependent transcription by targeting the TFIIIB complex. Mol Cell Biol *28*, 4204-4214.

Wu, J., Sun, L., Chen, X., Du, F., Shi, H., Chen, C., and Chen, Z.J. (2013). Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. Science *339*, 826-830.

Wu, L., Pan, J., Thoroddsen, V., Wysong, D.R., Blackman, R.K., Bulawa, C.E., Gould, A.E., Ocain, T.D., Dick, L.R., Errada, P., Dorr, P.K., Parkinson, T., Wood, T., Kornitzer, D., Weissman, Z., Willis, I.M., and McGovern, K. (2003). Novel small-molecule inhibitors of RNA polymerase III. Eukaryot Cell *2*, 256-264.

Xu, Y., and Komiyama, M. (2013). Evidence for G-Quadruplex DNA in Human Cells. Chembiochem.

Yanai, H., Ban, T., Wang, Z., Choi, M.K., Kawamura, T., Negishi, H., Nakasato, M., Lu, Y., Hangai, S., Koshiba, R., Savitsky, D., Ronfani, L., Akira, S., Bianchi, M.E., Honda, K., Tamura, T., Kodama, T., and Taniguchi, T. (2009). HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses. Nature *462*, 99-103.

Yang, Y.G., Lindahl, T., and Barnes, D.E. (2007). Trex1 exonuclease degrades ssDNA to prevent chronic checkpoint activation and autoimmune disease. Cell *131*, 873-886.

Yasutomo, K., Horiuchi, T., Kagami, S., Tsukamoto, H., Hashimura, C., Urushihara, M., and Kuroda, Y. (2001). Mutation of DNASE1 in people with systemic lupus erythematosus. Nat Genet *28*, 313-314.

Yi, R., Qin, Y., Macara, I.G., and Cullen, B.R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev *17*, 3011-3016.

Yonaha, M., Chibazakura, T., Kitajima, S., and Yasukochi, Y. (1995). Cell cycle-dependent regulation of RNA polymerase II basal transcription activity. Nucleic Acids Res *23*, 4050-4054.

Yoshida, H., Okabe, Y., Kawane, K., Fukuyama, H., and Nagata, S. (2005). Lethal anemia caused by interferon-beta produced in mouse embryos carrying undigested DNA. Nat Immunol *6*, 49-56.

Yuan, J., and Chen, J. (2010). MRE11-RAD50-NBS1 complex dictates DNA repair independent of H2AX. J Biol Chem *285*, 1097-1104.

Zaitsev, E.N., and Kowalczykowski, S.C. (2000). A novel pairing process promoted by Escherichia coli RecA protein: inverse DNA and RNA strand exchange. Genes Dev *14*, 740-749.

Zhang, X., Brann, T.W., Zhou, M., Yang, J., Oguariri, R.M., Lidie, K.B., Imamichi, H., Huang, D.W., Lempicki, R.A., Baseler, M.W., Veenstra, T.D., Young, H.A., Lane, H.C., and Imamichi, T. (2011a). Cutting edge: Ku70 is a novel cytosolic DNA sensor that induces type III rather than type I IFN. J Immunol *186*, 4541-4545.

Zhang, X., Wan, G., Berger, F.G., He, X., and Lu, X. (2011b). The ATM kinase induces microRNA biogenesis in the DNA damage response. Mol Cell *41*, 371-383.

Zhang, Z., Yuan, B., Bao, M., Lu, N., Kim, T., and Liu, Y.J. (2011c). The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells. Nat Immunol *12*, 959-965.

Zhao, J., Bacolla, A., Wang, G., and Vasquez, K.M. (2010). Non-B DNA structure-induced genetic instability and evolution. Cell Mol Life Sci *67*, 43-62.

Zhao, W. (2013). Negative regulation of TBK1-mediated antiviral immunity. FEBS Lett *587*, 542-548.

Appendices

Appendix A. Extract of Mass Spectrometry Mascot Search Results

a

gi|194381202 **Mass:** 48097 **Score:** 54 **Matches:** 2(1) **Sequences:** 2(1) **emPAI:** 0.07
unnamed protein product [Homo sapiens]
Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
750	613.8583	1225.7021	1225.7030	-0.73	0	54	9e-005	1	U	K.APILIATDVASR.G
957	678.8379	1355.6613	1355.6582	2.31	1	5	18	3	U	M.QFETTGSRFGAR.G

Proteins matching the same set of peptides:
gi|530419472 **Mass:** 48109 **Score:** 54 **Matches:** 2(1) **Sequences:** 2(1)
PREDICTED: probable ATP-dependent RNA helicase DDX17 isoform X2 [Homo sapiens]

b

AGO2_HUMAN **Mass:** 98400 **Score:** 35 **Matches:** 3(1) **Sequences:** 3(1) **emPAI:** 0.07
Protein argonaute-2 OS=Homo sapiens GN=AGO2 PE=1 SV=3
Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
502	527.3031	1052.5916	1052.5906	0.99	0	31	0.0043	1		R.ELLIQFYK.S
532	536.7890	1071.5634	1071.5713	-7.32	0	3	16	4	U	K.QFHTGIEIK.V
826	630.8251	1259.6357	1259.6358	-0.04	0	22	0.06	1	U	K.SGNIPAGTTVDTK.I

c

DDX17_HUMAN **Mass:** 80906 **Score:** 41 **Queries matched:** 2 **emPAI:** 0.04
Probable ATP-dependent RNA helicase DDX17 OS=Homo sapiens GN=DDX17 PE=1 SV=2
Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Peptide
610	590.2858	1178.5571	1178.5602	-2.58	0	1	7.1	2	R.DMVGIAQTGSGK.T + Oxidation (M)
663	613.8583	1225.7020	1225.7030	-0.82	0	41	0.00027	1	K.APILIATDVASR.G

Mascot Search Engine was used to analyze peptides detected from cutout gel regions of interest after A549 cytosolic lysate immunoprecipitation with either RNA:DNA hybrid antibodies or dsDNA antibodies. (a, b) Mass spectrometry analysis from Fig. 4.9 showing DDX17 and AGO2 as interacting proteins of RNA:DNA hybrids. (c) Mass spectrometry analysis from Fig. 3.10 identifies DDX17 as an interacting protein of dsDNA.